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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

In re Application of:	Art Unit: 1644
WALLACH et al	Examiner: R. Schwadron
Appln. No.: 08/485,129	Washington, D.C.
Filed: June 7, 1995	March 23, 2000
For: ISOLATED DNA ENCODING TUMOR NECROSIS FACTOR BINDING PROTEIN II, AND VECTORS, HOSTS AND PROCESSES USING SUCH DNA	Atty.Docket: WALLACH=5B

BRIEF ON APPEAL

Honorable Commissioner of Patents and Trademarks
Washington, D.C. 20231

Sir:

Submitted herewith is applicant's Brief on Appeal in triplicate.

The present appeal is taken from the action of the examiner in finally rejecting claims 11-13, 35-38, 43, 44, 46-49 and 51. The full text of claims 11-13, 35-38, 43, 44, 46-49 and 51 under appeal appears in Appendix A attached hereto.

REAL PARTY IN INTEREST

The present application is owned by Yeda Research and Development Co. Ltd., which is the research and development arm of the Weizmann Institute of Science in Rehovot, Israel. The exclusive licensee of the present invention is Inter-Lab Limited,

an Israeli company of Ness-Ziona, Israel. Inter-Lab Limited is a subsidiary of InterPharm Laboratories Limited, an Israeli company of Ness-Ziona, Israel, which is a subsidiary of Ares Serono N.V., whose parent company, Ares Serono S.A., is a holding company under which there are many subsidiaries worldwide.

RELATED APPEALS AND INTERFERENCES

Appellant is aware of no other appeals or interferences which will directly affect or be directly affected by or have a bearing on the Board's decision in the present appeal.

The present application is a divisional of parent application 07/930,443, filed August 19, 1992. All of the claims in the present case are drawn to invention(s) deleted from the parent application in light of a restriction requirement. The claims remaining in said parent application drawn to the TBP-II protein are now involved in an interference proceeding with the claims of U.S. patent 5,344,915. This is pending interference no. 103,625. While it is not believed that this interference will directly affect or be directly affected by or have a bearing on the Board's decision in the present appeal, it is nevertheless being brought to the Board's attention as it is related in the sense discussed above, and the examiner has required that the reference to this interference be made in the present section of the appeal.

STATUS OF CLAIMS

Claims 11-14, 35-39 and 43-51 presently appear in this case. Claims 11-13, 35-38, 43, 44, 46-49 and 51 are under final rejection. Claims 1-10, 15-34 and 40-42 have been cancelled. Claims 14, 39, 45 and 50 have been withdrawn from consideration, but it is understood that in the event that the claims on appeal are found allowable, these withdrawn claims will be treated as per MPEP §821.04.

STATUS OF AMENDMENTS

A final rejection was issued in this case on July 2, 1997. On November 3, 1997, an amendment after final was filed. By Advisory Action of December 2, 1997, the proposed amendment were entered. On March 31, 1998, another amendment after final rejection was filed, and on April 3, 1998, a further supplemental amendment after final rejection was filed. However, this latter supplemental amendment contained a request under 37 C.F.R. §1.129(a) that the finality of the official action of July 2, 1997, be withdrawn. By the official action of July 6, 1998, the examiner withdrew the finality of the previous office action and confirmed that all of applicants' amendments after final rejection of November 6, 1997, March 31, 1998, and April 3, 1998, had been entered.

On February 26, 1999, another final rejection was issued by the examiner. Subsequent to the final rejection of February 26, 1999, applicant filed an amendment on May 25, 1999, and a

supplemental communication submitting three certified Israel priority documents on July 8, 1999. By Advisory Action of August 20, 1999, the examiner indicated that upon filing of an appeal, the proposed amendment would be entered. The examiner also entered and considered the supplemental communication and the Israeli priority documents.

SUMMARY OF THE INVENTION

The present invention is directed to isolated DNA molecules which encode Tumor Necrosis Factor (TNF) Binding Protein II (TBP-II) (page 1, lines 2-6). The protein encoded by the DNA of the present invention was initially isolated from human urine and was found to have the ability of selectively inhibiting the cytotoxic effect of TNF (paragraph bridging pages 6 and 7). Under certain conditions it can also act as a carrier for TNF and thus maintain its prolonged beneficial effects (see page 6, lines 13-21, and Example 9, beginning at page 33).

This naturally occurring protein TBP-II, which was isolated from the urine, was found to include the following partial amino acid sequence: Thr-Pro-Tyr-Ala-Pro-Glu-Pro-Gly-Ser-Thr in the portion of the protein sequenced by N-terminal sequence analysis (see page 7, lines 13-15).

The TBP-II encoded by the DNA of the present invention derived from human urine concentrate showed an apparent molecular weight of 30 kD in reducing SDS-PAGE analysis (page 7, lines 2-3).

The DNA of the present invention may also encode active fractions of TBP-II provided the fraction has the ability to inhibit the cytotoxic effect of TNF (see page 15, lines 11-17).

The present claims are drawn to the isolated DNA molecules which encode the newly discovered TBP-II protein and active fragments thereof as well as replicable expression vehicles containing such DNA, host cells transformed with the replicable expression vehicle and processes for producing TBP-II by culturing such a transformant host cell (see page 8, lines 18-23, and claims 11-14 as originally filed).

One claim directed to the TBP-II protein was officially found to be allowable by the examiner in charge of the parent application. The claims drawn to the TBP-II protein in the parent application, 07/930,443, are now involved in an interference proceeding with the claims of U.S. patent 5,344,915.

THE PRIOR ART

The only prior art rejection in this case appearing in the final rejection of February 26, 1999, was withdrawn by the Advisory Action of August 20, 1999. Thus, there is no prior art which requires discussion in the present brief.

THE REJECTIONS

The rejection of claims 11-13 and 46-49 under 35 USC 112, first paragraph, as appearing in paragraph 17 of the final rejection was withdrawn in paragraph 2 of the Advisory Action of

August 20, 1999. The rejection of claims 35, 43 and 44 under 35 USC 112, first paragraph, as appearing in paragraph 18 of the final rejection was withdrawn in paragraph 3 of the Advisory Action of August 20, 1999. The rejection of claims 11-13, 35-38, 43, 44 and 46-49 under 35 USC 102(e) as appearing in paragraph 21 of the final rejection was withdrawn in paragraph 5 of the Advisory Action of August 20, 1999. Thus, the only rejection remaining in this case for review in the present appeal is the rejection in paragraph 19 of the final rejection which was repeated in paragraph 4 of the Advisory Action of August 20, 1999. The examiner's restatement of the rejection in this Advisory Action and his comments in response to applicants' arguments, which were not deemed persuasive, are as follows:

> Claims 11-13, 35-38, 43, 44, 46-49, 51 stand rejected under 35 U.S.C. 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention for the reasons elaborated in the previous Office Action. Applicants arguments have been considered and deemed not persuasive.
>
> The specification does not provide adequate written description of the claimed invention. The legal standard for sufficiency of a patent's (or a specification's) written description is whether that description "reasonably conveys to the artisan that the inventor had possession at that time of the ... claimed subject matter", Vas-Cath, Inc. V. Mahurkar, 19 U.S.P.Q.2d 1111 (Fed. Cir. 1991). In the instant case, the specification does not convey to the artisan that the applicant had possession at the time of invention of the claimed DNAs and molecules containing said DNAs.

The instant claims encompass an isolated DNA molecule or vectors or host cells which contain said DNA wherein said DNA encodes a protein consisting of naturally occurring TBP-II. There is no disclosure in the specification of an intact DNA sequence which encodes said molecule. There is no disclosure in the specification of any DNA sequence which encodes the claimed DNA. The claimed molecule recites physical features of a TBP-II protein and the amino acid sequences of a 10-13 amino acid sequence of the N terminal of a molecule that contains at least 250 amino acids. There is no disclosure in the specification of any DNA sequence which encodes the claimed molecule. In view of the aforementioned problems regarding description of the claimed invention, the specification does not provide an adequate written description of the invention claimed herein. See The Regents of the University of California v. Eli Lilly and Company, 43 USPQ2d 1398,1404-7 (Fed. Cir. 1997). In University of California v. Eli Lilly and Co., 39 U.S.P.Q.2d 1225 (Fed. Cir. 1995) the inventors claimed a genus of DNA species encoding insulin in different vertebrates or mammals, but had only described a single species of cDNA which encoded rat insulin. The court held that only the nucleic acids species described in the specification (i.e. nucleic acids encoding rat insulin) met the description requirement and that the inventors were not entitled to a claim encompassing a genus of nucleic acids encoding insulin from other vertebrates, mammals or humans, id. at 1240. In the instant case, the specification has not provided even a single DNA sequence which encodes the claimed DNA. The Federal Circuit has held that if an inventor is "unable to envision the detailed constitution of a gene so as to distinguish it from other materials ... conception has not been achieved until reduction to practice has occurred", Amgen, Inc. v. Chugai Pharmaceutical Co, Ltd., 18 U.S.P.Q.2d 1016 (Fed. Cir. 1991). Attention is also directed

to the decision of The Regents of the University of California v. Eli Lilly and Company (CAFC, July 1997) wherein is stated: The description requirement of the patent statute requires a description of an invention, not an indication of a result that one might achieve if one made that invention. See In re Wilder, 736 F.2d 1516, 222 USPQ 369, 372-373 (Fed. Cir. 1984) (affirming rejection because the specification does "little more than outlin[e] goals appellants hope the claimed invention achieves and the problems the invention will hopefully ameliorate."). Accordingly, naming a type of material generally known to exist, in the absence of knowledge as to what that material consists of, is not a description of that material.

Thus, as we have previously held, a cDNA is not defined or described by the mere name "cDNA," even if accompanied by the name of the protein that it encodes but requires a kind of specificity usually achieved by means of the recitation of the sequence of nucleotides that make up the cDNA. See Fiers, 984 F.2d at 1171, 25 USPQ2d at 1606.

Regarding applicants comments in the instant amendment about the criterion C(2) from the interim written description guidelines, the following comments are made. The particular paragraph from C(2) which applicant quotes on page 14 of the instant amendment indicates that in order to meet the written description requirement the characteristics of the claimed invention need to be described "in such full, clear concise and exact terms that a skilled artisan would recognize applicant was in possession of the claimed invention". In the instant application, while applicant has disclosed information and methods to obtain the claimed nucleic acid sequence, applicant was clearly not in possession of the claimed invention at the time the instant application was filed. There is no disclosure in the specification of isolated nucleic acids encoding the molecule recited in the claims.

Regarding the particular example from the interim written description guidelines which applicant quotes in page 15 of the instant amendment, said example differs from the instant application in that the example discloses a scenario wherein the applicant was in physical possession of the claimed molecule. In order to know that said molecule had the particular characteristics disclosed in said example, the molecule was isolated and demonstrated to have said characteristics. Therefore, applicant had physical possession of said molecule. It would be impossible to know the restriction and/or nuclease cleave sites without knowing the intact sequence of said nucleic acid or without having physically isolated the nucleic acid and empirically determined the information. In the case of the instant application, applicant has not demonstrated possession of the claimed invention because while applicant has disclosed a method for isolating said molecule, the molecule was not isolated. Similarly, regarding the enzyme example listed in page 16 of the instant amendment, in order to determine the various physical properties recited in said claim, it was necessary to have already obtained and possessed said molecule. In the case of the instant application, applicant has not demonstrated possession of the claimed invention because while applicant has disclosed a method for isolating said molecule, the molecule was not isolated. Thus, the instant claims do not meet the criterion section C(2) from the interim written description guidelines. Regarding applicants theory that disclosure of a protein provides written description of the nucleic acid, there is no disclosure in the instant application of the amino acid sequence of TBP-II.

Regarding applicants comments in the instant amendment about University of California v. Eli Lilly, there is still no disclosure in the specification of any nucleic acid encoding the scope of the claimed invention (eg. a nucleic

acid encoding TBP-II). There is also no disclosure in the specification of the amino acid sequence of intact TBP-II. While the specification discloses N-terminal amino acid sequence data indicating a possible partial amino acid sequence of 31 amino acids of TBP-II, said peptide contains at least 250 amino acids, wherein the identity of the vast majority of said amino acids has not been disclosed in the specification. In University of California v. Eli Lilly, the court held that only the nucleic acids species described in the specification (i.e. nucleic acids encoding rat insulin) met the description requirement and that the inventors were not entitled to a claim encompassing a genus of nucleic acids encoding insulin from other vertebrates, mammals or humans, Id. at 1240. In the instant case, the specification has not provided even a single DNA sequence which encodes the claimed DNA. The Federal Circuit has held that if an inventor is "unable to envision the detailed constitution of a gene so as to distinguish it from other materials ... conception has not been achieved until reduction to practice has occurred", Amgen, Inc. v. Chugai Pharmaceutical Co, Ltd., 18 U.S.P.Q.2d 1016 (Fed. Cir. 1991). Attention is also directed to the decision of The Regents of the University of California v. Eli Lilly and Company (CAFC, July 1997) wherein is stated: The description requirement of the patent statute requires a description of an invention, not an indication of a result that one might achieve if one made that invention. See In re Wilder, 736 F.2d 1516, 222 USPQ 369, 372-373 (Fed. Cir. 1984) (affirming rejection because the specification does "little more than outlin[e] goals appellants hope the claimed invention achieves and the problems the invention will hopefully ameliorate."). Accordingly, naming a type of material generally known to exist, in the absence of knowledge as to what that material consists of, is not a description of that material.

Regarding applicants comments that TBP-II protein is disclosed in the specification and the intact amino acid sequence of TBP-II could

be obtained using the methods disclosed in the specification, this is not the issue under consideration. The Federal Circuit has held that if an inventor is "unable to envision the detailed constitution of a gene so as to distinguish it from other materials ... conception has not been achieved until reduction to practice has occurred", Amgen, Inc. v. Chugai Pharmaceutical Co, Ltd., 18 U.S.P.Q.2d 1016 (Fed. Cir. 1991). Clearly, in the instant application, the inventor is unable to envision the detailed constitution of a nucleic acid so as to distinguish it from other materials because the sequence of the claimed nucleic acid was not known to the inventors at the time of the filing date of the instant application. Regarding applicants comments about the TBP-II protein, none of the claims of the instant invention are drawn to TBP-II protein. The claims under consideration are drawn to nucleic acids. The possession of an isolated protein in itself provides no written description of the identity of the nucleic acid encoding said protein in the absence of the complete amino acid sequence of said protein. Applicants response recites "Once the complete amino acid sequence is known, all contiguous DNA sequences which encode such a protein are known in view of the known rules of the genetic code.". However, the complete amino acid sequence of TBP-II is not disclosed in the instant application. The instant application merely recites methods that could be potentially used to elucidate the nature of said sequence. In the absence of the disclosure of the claimed nucleic acid in the specification or the complete amino acid sequence of TBP-II there is no written description of the scope of the claimed invention. Regarding applicants comments that University of California v. Eli Lilly only applies to "genes" per se, this not stated in University of California v. Eli Lilly. In fact, in University of California v. Eli Lilly the court clearly states that:

> *An adequate written description of a DNA, such as the cDNA of the recombinant plasmids and microorganisms of the '525 patent, "requires a precise definition, such as by structure, formula, chemical name, or physical properties," not a mere wish or plan for obtaining the claimed chemical invention. Fiers v. Revel, 984 F.2d 1164, 1171, 25 USPQ2d 1601, 1606 (Fed. Cir. 1993). Accordingly, "an adequate written description of a DNA requires more than a mere statement that it is part of the invention and reference to a potential method for isolating it; what is required is a description of the DNA itself." Id. at 1170, 25 USPQ2d at 1606.*
>
> In the instant application, applicants has provided a plan and potential method for isolating the claimed nucleic acids, but have provided no written description of said nucleic acids.

ISSUE

The following issue is presented in this appeal:

Is there adequate written description for a claim covering all DNA sequences which encode a novel isolated protein defined by a partial amino acid sequence and other characterizing features?

GROUPING OF THE CLAIMS

All of the claims stand or fall together

ARGUMENT

The Patent Specification as Filed Describes the Claimed DNA in Sufficient Detail that One Skilled in the Art Can Reasonably Conclude that the Inventor Had Possession of the Claimed DNA

Applicant's position with respect to the written description rejection can be thumbnailed by the following syllogism.

1. The specification contains adequate written description for the TBP protein.

2. The complete amino acid sequence of a protein is an inherent property of an isolated protein. Therefore, even though the complete amino acid sequence was not explicitly disclosed, applicant was inherently in possession of the complete amino acid sequence.

3. Once one has demonstrated possession of the complete amino acid sequence, the genetic code automatically puts one in possession of all DNA sequences encoding that amino acid sequence. QED.

As to the first paragraph of the above syllogism, it is not believed that the examiner disputes the fact that there is written description for the TBP protein in the application as originally filed. It should be noted that during the prosecution of this case the examiner has not refuted this particular part of the syllogism. The examiner states at the beginning of the first full paragraph on page 4 of the Advisory Action of August 20, 1999:

> Regarding applicant's comments that TBP-II protein is disclosed in the specification and the intact amino acid sequence of TBP-II could

> be obtained using the method disclosed in the specification, this is not the issue under consideration.

The first paragraph of the syllogism is consistent with the Revised Interim Guidelines.[1] Section II.3.A.(1)(a)-(c) of these Guidelines states that, for original claims, for each claim drawn to a single species, one must first determine whether the application describes an actual reduction to practice of the claimed invention or if there is evidence of a completed invention by reduction to drawings. Section (c) goes on to state:

> If the application does not describe an actual reduction to practice or reduction to drawings, determine whether the invention has been set forth in terms of distinguishing identifying characteristics as evidenced by other descriptions of the invention that are sufficiently detailed to show that applicant was in possession of the claimed invention.

Here, with respect to the protein, there was an actual reduction to practice as the protein was actually isolated. Furthermore, the protein was described by a partial amino acid sequence and sufficient other distinguishing identifying characteristics that are sufficiently detailed to show that applicant was in possession of the claimed invention. The fact that the protein was adequately described to comply with the written description requirement is evidenced by the fact that at

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[1] Throughout this brief, "Revised Interim Guidelines" will refer to the "Revised Interim Guidelines for Examination of Patent Applications under the 35 U.S.C. §112, §1 'Written Description' Requirement", published in the Federal Register on December 21, 1999, at 64(244) 71427-71440. A copy of the Revised Interim Guidelines is attached hereto as Appendix B for the convenience of the Board.

least one protein claim was allowed during the prosecution of the parent application 07/930,443, which is now involved in an interference proceeding with the claims of U.S. patent 5,344,915.

With respect to the second paragraph of the above syllogism, reference is made to Ex parte Yamaguchi, 6 USPQ2d 1805, 1807 (BdPatApp&Int 1987), where it states:

> Moreover, it is well settled that from a standpoint of patent law, a compound and all of its properties are inseparable. They are one and the same. *In re Papesch* 50, CCPA 1084, 315 F2d 381, 137 USPQ 43 (1963). In our view, the X-ray diffraction spectrum, like the graphic formulae, the chemical nomenclature, etc., is merely a symbol by which the compounds can be identified, classified and compared.

The same is true for the amino acid sequence of a protein.

See also Ex parte Marsili, 214 USPQ 904 (PTOBdApp 1979) which held that a change in the structural formula of a chemical compound that was adequately described in terms of its characteristics in the original specification did not violate the description requirement. It is also noted that in the Board decision of Ex parte Deuel, 27 USPQ2d 1360, 1363 (BdPatApp&Int 1993), the Board noted the examiner's position that the amino acid sequence is an inherent characteristic of the protein.

In this case, in light of the partial amino acid sequence of the protein and the other characterizing features disclosed, as well as the method for obtaining the protein, one of ordinary skill in the art could obtain the entire amino acid sequence of the protein without undue experimentation.

As to the third paragraph of the syllogism, it is clear that the present DNA claims generically encompass all DNA sequences encoding naturally occurring human TBP-II. As the genetic code provides a direct relationship of amino acid sequences and associated nucleic acid codons, it is a scientific fact that given the complete amino acid sequence of a protein, coupled with knowledge of the genetic code, one is in possession of the genus of all of the DNA sequences which will encode that complete amino acid sequence. In re Deuel, 34 USPQ2d 1210, 1216 (Fed. Cir. 1995), noted that, with the aid of a computer, a person of ordinary skill in the art may even be able to identify all members of the claimed genus. Thus, if one is in possession of the complete amino acid sequence encoded by a claimed DNA sequence, then one is necessarily in possession of the entire claimed DNA genus.

As stated in the Revised Interim Guidelines in the second paragraph of Section I:

> To satisfy the written description requirement, a patent specification must describe the claimed invention in sufficient detail that one skilled in the art can reasonably conclude that the inventor had possession of the claimed invention.

Similarly, the last paragraph of Section I reads:

> The fundamental factual inquiry is whether the specification conveys with reasonable clarity to those skilled in the art that, as of the filing date sought, applicant was in possession of the invention as now claimed.

Thus, it is possession of the claimed invention which is important. The above syllogism establishes that applicant was in

possession of the genus of DNA sequences that encode the single species of naturally occurring human TBP-II. As applicant was in possession of the invention as now claimed, the fundamental factual inquiry necessary to satisfy the written description requirement must be answered in the affirmative.

There is nothing in the case law cited by the examiner which precludes an applicant from claiming the genus of DNA which encodes an adequately disclosed protein. Admittedly, if the present claims were directed to the human cDNA encoding TBP-II, the case law would require a 35 USC 112, first paragraph, written description rejection because it would have been impossible for applicant to envision that single specific sequence which is the cDNA. Thus, even though there is written description in the present specification for the genus of all DNA sequences which encode a given amino acid sequence, there is admittedly no written description for the specific species of the cDNA, and indeed the specific species of the cDNA is not being specifically claimed in the present application. In the case relied upon by the examiner, discussed in detail hereinbelow, the claims being reviewed for compliance with the written description requirement were directed to the cDNA and not to broad DNA claims covering any DNA sequence which encodes the novel protein. Indeed, in the case cited by the examiner, the protein was not novel and therefore a generic DNA claim, such as is presently claimed, would have been obvious.

More specifically, the examiner relies mainly on University of California v. Eli Lilly and Co., 43 USPQ2d 1398 (Fed. Cir. 1997). While that case relates to the infringement of

two patents, i.e., patents 4,652,525 and 4,431,740 owned by the Regents of the University of California (UC), validity issues relating to the written description requirement of the first paragraph of 35 USC 112 were raised only with respect to the '525 patent. Copies of the front page and claims of these two patents are attached hereto as Appendices C and D. It can be seen that, in the '525 patent, all of the claims are directed to insulin-encoding cDNA, or the reverse transcript of mRNA which encodes insulin, which is synonymous with cDNA. Note that the Federal Circuit in the Lilly case at page 1405 characterizes claims 1 and 2 of the '525 patent as being claims "which *generically* recite cDNA encoding vertebrate insulin, and claim 4, which is directed *generically* to cDNA encoding mammalian insulin" [emphasis original] and that dependent claims 6 and 7 "similarly recite cDNA encoding vertebrate insulin." As to claim 5, the court stated, at pages 1404-1405:

> Claim 5 is directed to a recombinant prokaryotic microorganism modified so that it contains "a nucleotide sequence having the structure of the reverse transcript of an mRNA of a [human], which mRNA encodes insulin.

Thus, the definition of the claimed microorganism is one that requires human insulin-encoding cDNA. The validity of claim 3 was not before the court. Thus, it is very clear that all of the claims being construed for compliance with the written description requirement were claims directed to cDNA, i.e., the naturally occurring sequence which is only one of the myriad of possible sequences which encode human insulin due to the degeneracy of the genetic code. Therefore, the holdings in the Lilly case which

require that the sequence of the cDNA be known before that cDNA can be in the possession of the inventors so as to satisfy the written description requirement, are all related to the specific situation before the court in which all that is being claimed is cDNAs, either a cDNA of a single species or a genus of cDNAs of a plurality of animal species.

In the Advisory Action of August 20, 1999, in response to applicant's previous arguments that the Lilly case applied only to cDNAs *per se*, the examiner refers to page 1404 of Lilly where it states:

> An adequate written description of a DNA, such as the cDNA of the recombinant plasmids and microorganisms of the '525 patent, "requires a precise definition, such as by structure, formula, chemical name, or physical properties," not a mere wish or plan for obtaining the claimed chemical invention.

However, when the DNA claim is written broadly so as to include all DNA which encodes a particular amino acid sequence, the description of the amino acid sequence or sufficient characterizing information to establish that applicant was in possession of a novel protein, is sufficient to satisfy the requirement for a precise definition. Indeed, the examiner himself states at page 6 of the Advisory Action:

> In the absence of the disclosure of the claimed nucleic acid in the specification, or the complete amino acid sequence of TBP-II there is no written description of the scope of the claimed invention. [Emphasis added]

Thus, the examiner appears to admit that if applicant were in possession of the complete amino acid sequence of TBP-II, then

applicant would automatically be in possession of the claimed nucleic acid sequence which anyone of ordinary skill in the art could write as a formula once the complete amino acid sequence is known. Indeed, reference is made to claim 5 of the '740 patent involved in the Lilly case in which such a DNA sequence broad enough to encompass all DNAs which encode human proinsulin is set forth. Such a formula can readily be prepared for any given amino acid sequence without any knowledge of the naturally occurring cDNA.

Reference is also made to footnote 13 of the Revised Interim Guidelines which explicitly states:

> [a] genetic code table would correlate a known amino acid sequence with a genus of coding nucleic acids ... .

Here, applicant readily admits that the specification does not contain a complete amino acid sequence of TBP-II. However, it does disclose a partial amino acid sequence and sufficient other characterizing features to establish that applicant was in possession of the protein. Indeed, applicant had isolated the protein. The written description requirement was satisfied for the protein as is evidenced by the allowability of at least one protein claim in the parent application. As the complete amino acid sequence of a protein is an inherent property of an adequately described protein which is in possession of the applicant and a genetic code table can correlate any amino acid sequence with a genus of coding nucleic acids, it must necessarily follow that adequate written description of a protein is

inherently an adequate written description of a broad DNA claim which encompasses all nucleotide sequences which encode that protein.

There is nothing in the Revised Interim Guidelines which mandates a rejection of the present claims under the written description requirement. Indeed, in the response to comment 6 at page 71429 of the Federal Register notice, the material accompanying these Guidelines makes clear that the Revised Interim Guidelines do not impose a *per se* requirement for reduction to practice in any technology to satisfy the written description requirement. The discussion goes on to state:

> However, the Federal Circuit has recognized that in some instances an inventor may only be able to establish a conception (and therefore possession) by pointing to a reduction to practice through a successful experiment. ... In such instances, the alleged conception fails not merely because the field is unpredictable or because of the general uncertainty surrounding experimental sciences, but because the conception is incomplete due to factual uncertainty that undermines the specificity of the inventor's idea of the invention.

Here, while applicant may not have reduced to practice a specific DNA, applicant has reduced to practice the protein. Applicant has possession of the protein and has provided adequate written description of the protein. The complete amino acid sequence of the protein is an inherent property of the protein. Because the formula of all DNA which encompass that amino acid sequence is dictated by the genetic code, i.e., is a fixed formula, the DNA sequence is as much an inherent property of the adequately

described protein which has been reduced to practice as is the complete amino acid sequence thereof. Therefore, there is no actual uncertainty that undermines the specificity of the inventor's idea of the invention, such as would require an actual reduction to practice of a DNA before an applicant can be in possession thereof.

The statement in Section II.A.3 at the right column of page 71435 of the Federal Register notice is also applicable where it states:

> An applicant may also show that an invention is complete by disclosure of sufficiently detailed relevant identifying characteristics which provide evidence that applicant was in possession of the claimed invention, *i.e.*, complete or partial structure, other physical and/or chemical properties, functional characteristics when coupled with a known or disclosed correlation between function and structure, or some combination of such characteristics.

Such sufficiently detailed relevant identifying characteristics have been provided in the present specification for the protein. As the complete amino acid sequence of the protein is an inherent characteristic of the protein and as the formula for DNA which encodes the complete amino acid sequence is a fixed formula determined by the genetic code, such DNA formula is also an inherent characteristic of the adequately described protein.

Furthermore, the claims effectively include a partial nucleic acid sequence. All of the present claims recite at least 10 amino acid residues of the protein encoded by the DNA. Thus, at least 30 nucleotides of the DNA are disclosed. Regardless of the fact that the DNA molecule of the present invention is much

longer than 30 nucleotides, this is an important unique bit of characterizing information. This piece of nucleotide structure, in conjunction with the characterizing information that the DNA encodes a protein having the ability to inhibit the cytotoxic effects of TNF, provides sufficient relevant identifying characteristics to comply with the criteria of the above-quoted portion of the Revised Interim Guidelines.

The same paragraph of the Revised Interim Guidelines goes on to state:

> If a skilled artisan would have understood the inventor to be in possession of the claimed invention at the time of filing, even if every nuance of the claims is not explicitly described in the specification, then the adequate description requirement is met.

This sentence further supports the conclusion reached by the syllogism set forth hereinabove. Accordingly, for the reasons discussed in detail hereinabove, possession of a novel protein and a written description thereof sufficient to comply with the written description requirement of the first paragraph of 35 USC 112 inherently places one in possession of the formula of all DNA which encodes that protein. As the complete amino acid sequence of that protein is an inherent property of the protein and the generic DNA sequence which encodes that amino acid sequence is directly correlatable therewith by means of a genetic code table, a holding that the present claims comply with the written description requirement would be fully consistent with the newly issued Revised Interim Guidelines. Reversal of the examiner and withdrawal of this rejection are therefore respectfully urged.

CONCLUSION

The claims as submitted are believed to truly set forth the inventive concept of the present invention and to fully comply with the written description requirement of the first paragraph of 35 USC 112. Accordingly, reversal of the examiner and allowance of claims 11-13, 35-38, 43, 44, 46-49 and 51 are earnestly solicited.

Respectfully submitted,

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APPENDIX A

11. An isolated DNA molecule comprising a contiguous nucleotide sequence coding for a protein consisting of naturally occurring human Tumor Necrosis Factor (TNF) Binding Protein II, herein designated TBP-II, said TBP-II including the amino acid sequence: Thr-Pro-Tyr-Ala-Pro-Glu-Pro-Gly-Ser-Thr in the portion of the protein sequenced by N-terminal sequence analysis, said protein having the ability to inhibit the cytotoxic effect of TNF, wherein said naturally occurring TBP-II protein is the same as that protein having the ability to inhibit the cytotoxic effect of TNF which, after being purified by subjecting a crude protein recovered from a dialyzed concentrate of human urine to affinity chromatography on a column of immobilized TNF, elutes from a reversed-phase high pressure liquid chromatography column as a single peak in a fraction corresponding to about 31% acetonitrile and shows a molecular weight of about 30 kDa when measured by SDS-PAGE under reducing conditions.

12. A replicable expression vehicle comprising the DNA molecule of claim 11 and capable, in a transformant host cell, of expressing said protein.

13. A host cell selected from the group consisting of a prokaryotic and a eukaryotic cell transformed with the replicable expression vehicle of claim 12.

35. An isolated DNA molecule in accordance with claim 51, comprising

(1) the nucleotide sequence coding for a naturally occurring human Tumor Necrosis Factor (TNF) binding protein (TBP-II) having the following characteristics:

i. includes the amino acid sequence Thr-Pro-Tyr-Ala-Pro-Glu-Pro-Gly- Ser-Thr in the portion of the protein sequenced by N-terminal sequence analysis; and

ii. the ability to inhibit the cytotoxic effect of TNF-α on murine A9 cells, or

(2) a contiguous nucleotide sequence coding for a fragment of said TBP-II which has the ability to inhibit the cytotoxic effect of TNF-α on murine A9 cells.

36. An isolated DNA molecule comprising

(1) the nucleotide sequence coding for a naturally occurring human Tumor Necrosis Factor (TNF) binding protein (TBP-II) having the following characteristics:

i. includes the amino acid sequence Thr-Pro-Tyr-Ala-Pro-Glu-Pro-Gly- Ser-Thr in the portion of the protein sequenced by N-terminal sequence analysis; and

ii. the ability to inhibit the cytotoxic effect of TNF-α on murine A9 cells; and

iii. a molecular weight of about 30kd in reducing SDS-PAGE analysis, or

(2) a contiguous nucleotide sequence coding for a fragment of said TBP-II which has the ability to inhibit the cytotoxic effect of TNF-α on murine A9 cells.

37. A replicable expression vehicle comprising the DNA molecule of claim 51 and capable, in a transformant host cell, of expressing said protein.

38. A host cell selected from the group consisting of a prokaryotic and a eukaryotic cell transformed with the replicable expression vehicle of claim 37.

43. A replicable expression vehicle comprising the DNA molecule of claim 35 and capable, in a transformant host cell, of expressing said protein.

44. A host cell selected from the group consisting of a prokaryotic and a eukaryotic cell transformed with the replicable expression vehicle of claim 43.

46. An isolated DNA molecule comprising (1) a contiguous nucleotide sequence coding for a protein consisting of naturally occurring human Tumor Necrosis Factor (TNF) Binding Protein II, herein designated TBP-II, said TBP-II including the amino acid sequence: Thr-Pro-Tyr-Ala-Pro-Glu-Pro-Gly-Ser-Thr in

the portion of the protein sequenced by N-terminal sequence analysis, said protein having the ability to inhibit the cytotoxic effect of TNF, wherein said naturally occurring TBP-II protein is the same as that protein having the ability to inhibit the cytotoxic effect of TNF which, after being purified by subjecting a crude protein recovered from a dialyzed concentrate of human urine to affinity chromatography on a column of immobilized TNF, elutes from a reversed-phase high pressure liquid chromatography column as a single peak in a fraction corresponding to about 31% acetonitrile and shows a molecular weight of about 30 kDa when measured by SDS-PAGE under reducing conditions, or (2) a contiguous nucleotide sequence coding for a fragment of said TBP-II which has the ability to inhibit the cytotoxic effect of TNF.

47. An isolated DNA molecule in accordance with claim 51, wherein said nucleotide sequence is the sequence of (2).

48. A replicable expression vehicle comprising the DNA molecule of claim 47 and capable, in a transformant host cell, of expressing said protein.

49. A host cell selected from the group consisting of a prokaryotic and a eukaryotic cell transformed with the replicable expression vehicle of claim 48.

51. An isolated DNA molecule comprising

(1) a contiguous nucleotide sequence coding for a naturally occurring human Tumor Necrosis Factor (TNF) binding protein (TBP-II) having the following characteristics:

(a) includes the amino acid sequence Thr-Pro-Tyr-Ala-Pro-Glu-Pro-Gly-Ser-Thr in the portion of the protein sequenced by N-terminal sequence analysis; and

(b) has the ability to inhibit the cytotoxic effect of TNF; or

(2) a contiguous nucleotide sequence coding for a fragment of said TBP-II which has the ability to inhibit the cytotoxic effect of TNF.

roger\briefs\wal15b.clm

FOR FURTHER INFORMATION CONTACT: Dan Waldeck, Pacific Fishery Management Council; (503) 326–6352.

SUPPLEMENTARY INFORMATION: The primary purpose of the work session is to draft sections of the fishery management plan and related documents for highly migratory species fisheries off the West Coast.

Although non-emergency issues not contained in the HMSPDT meeting agenda may come before the HMSPDT for discussion, those issues may not be the subject of formal HMSPDT action during these meetings. HMSPDT action will be restricted to those issues specifically listed in this notice and any issues arising after publication of this notice that require emergency action under section 305(c) of the Magnuson-Stevens Fishery Conservation and Management Act, provided the public has been notified of the HMSPDT's intent to take final action to address the emergency.

Special Accommodations

The meeting is physically accessible to people with disabilities. Requests for sign language interpretation or other auxiliary aids should be directed to Mr. John Rhoton at (503) 326–6352 at least 5 days prior to the meeting date.

Dated: December 16, 1999.

Bruce C. Morehead,

Acting Director, Office of Sustainable Fisheries, National Marine Fisheries Service.

[FR Doc. 99–33066 Filed 12–20–99; 8:45 am]

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DEPARTMENT OF COMMERCE

Patent and Trademark Office

[Docket No. 991027288–9288–01]

RIN 0651–AB10

Revised Interim Guidelines for Examination of Patent Applications Under the 35 U.S.C. § 112, ¶ 1 "Written Description" Requirement; Request for Comments

AGENCY: Patent and Trademark Office, Commerce.

ACTION: Notice and request for public comments.

SUMMARY: The Patent and Trademark Office (PTO) requests comments from any interested member of the public on the following Revised Interim Guidelines for Examination of Patent Applications Under the 35 U.S.C. 112, ¶ 1 "Written Description" Requirement (Revised Interim Guidelines). These Revised Interim Guidelines will be used by PTO personnel in their review of patent applications for compliance with the "written description" requirement of 35 U.S.C. § 112,¶ 1. This revision supersedes the Interim Written Description Guidelines which were published contemporaneously in both the **Federal Register** and Official Gazette at 63 FR 32,639 (June 15, 1998) and 1212 O.G. 15 (July 7, 1998), respectively. This revision reflects the current understanding of the PTO regarding the written description requirement of 35 U.S.C. 112, ¶ 1 and is applicable to all technologies.

DATES: Written comments on the Revised Interim Guidelines will be accepted by the PTO until March 22, 2000.

ADDRESSES: Written comments should be addressed to Box 8, Commissioner of Patents and Trademarks, Washington, DC 20231, marked to the attention of Stephen Walsh, or to Box Comments, Assistant Commissioner for Patents, Washington, DC 20231, marked to the attention of Linda S. Therkorn. Alternatively, comments may be submitted to Stephen Walsh via facsimile at (703) 305–9373 or by electronic mail addressed to "stephen.walsh@uspto.gov" or to Linda Therkorn via facsimile at (703) 305–8825 or by electronic mail addressed to "linda.therkorn@uspto.gov."

FOR FURTHER INFORMATION CONTACT: Stephen Walsh by telephone at (703) 305–9035, by facsimile at (703) 305–9373, by mail to his attention addressed to Box 8, Commissioner of Patents and Trademarks, Washington, DC 20231, or by electronic mail at "stephen.walsh@uspto.gov"; or Linda Therkorn by telephone at (703) 305–8800, by facsimile at (703) 305–8825, by mail addressed to Box Comments, Assistant Commissioner for Patents, Washington, DC 20231, or by electronic mail at "linda.therkorn@uspto.gov."

SUPPLEMENTARY INFORMATION: The PTO requests comments from any interested member of the public on the following Revised Interim Guidelines. As of the publication date of this notice, this revision will be used by PTO personnel in their review of patent applications for compliance with the "written description" requirement of 35 U.S.C. 112, ¶ 1. Because this revision governs internal practices, it is exempt from notice and comment rulemaking under 5 U.S.C. 553(b)(A).

Written comments should include the following information: (1) Name and affiliation of the individual responding, and (2) an indication of whether the comments offered represent views of the respondent's organization or are respondent's personal views. If you believe the PTO should further amend these revised interim guidelines before they are made final, you should include the following information in your comments: (1) The rationale supporting the proposal, including the identification of applicable legal authority; and (2) a description of the potential benefits and drawbacks of adopting the proposal. The PTO is particularly interested in comments relating to the following topics: (1) The accuracy of the methodology, (2) the legal analysis in the guidelines, and (3) relevant factors to consider in determining whether the written description requirement is satisfied.

Parties presenting written comments are requested, where possible, to provide their comments in machine-readable format in addition to a paper copy. Such submissions may be provided by electronic mail messages sent over the Internet, or on a 3.5" floppy disk formatted for use in a Macintosh, Windows, Windows for Workgroups, Windows 95, Windows 98, Windows NT, or MS–DOS based computer.

Written comments will be available for public inspection on or about April 19, 2000, in Suite 918, Crystal Park 2, 2121 Crystal Drive, Arlington, Virginia. In addition, comments provided in machine readable format will be available through the PTO's Website at http://www.uspto.gov.

Discussion of Public Comments

Comments were received from 13 individuals and 16 organizations in response to the Request for Comments on the Interim Guidelines for the Examination of Patent Applications Under the 35 U.S.C. 112, ¶ 1 "Written Description" Requirement published contemporaneously in the **Federal Register** and Official Gazette at 63 FR 32,639 (June 15, 1998) and 1212 O.G. 15 (July 7, 1998), respectively; and the Extension of Comment Period and Notice of Hearing published at 63 FR 50887 (September 23, 1998) and 1214 O.G. 180 (September 29, 1998). The written comments and the testimony at the public hearing have been carefully considered.

Overview of Comments

The majority of comments favored issuance of written description guidelines, with revisions. Several major issues arose in the oral testimony and written comments submitted in response to the Interim Guidelines on the Written Description Requirement with respect to the scope of the Guidelines, the method of analysis, and the content of the examples. In view of

the comments and testimony received, the Guidelines have been rewritten in a technology neutral manner which is broadly applicable to all areas of technology and to all types of claims (original, new, or amended, and product, process, or product-by-process). Furthermore, the examples have been removed from the Guidelines and examples addressing a broad range of technologies will be incorporated into examiner training materials. Revised Interim Guidelines are being issued for a second round of Notice and Comment because the form and content of the Guidelines are sufficiently different from the previous Guidelines that additional public comment is desired.

The Extension of Comment Period and Notice of Hearing published at 63 FR 50887 (September 23, 1998) and 1214 O.G. 180 (September 29, 1998) asked for comments regarding the patentability of Expressed Sequence Tags (ESTs). Many comments took this opportunity to heavily criticize the patentability of ESTs, grounding their arguments in fairness and policy issues. Many comments also expressed the opinion that ESTs lacked the utility, enablement, and written description necessary to satisfy title 35 of the U.S. Code. The Revised Interim Guidelines are not the appropriate vehicle to fully address the patentability of ESTs. In view of comments and testimony with respect to ESTs and the enablement and utility requirements, the Office is revising the Utility Guidelines as published at 60 FR 36263 (July 14, 1995), and will also be revising the examiner training material with regard to both the utility and enablement requirements. Comments pertaining to the utility and enablement requirements will be addressed in the notice revising the Utility Guidelines. Responses to the comments germane to the written description requirement are set forth below.

Responses to Specific Comments

(1) *Comment:* Several comments criticized the Guidelines for failing to set out a general, systematic examination of the case law on written description. Comments mentioned *Vas-Cath, Inc.* v. *Mahurkar*, 935 F.2d 1555, 19 USPQ2d 1111 (Fed. Cir. 1991), in particular as important for summarizing the state of the law as the Federal Circuit sees it. Other comments particularly urged a general analysis of case law as it pertains to written description for chemical compounds, and criticized the fact that the Guidelines relied heavily on only three recent cases. *Response:* The suggestion to provide a general, systematic legal analysis has been adopted. The Revised Interim Guidelines are grounded more broadly than the three cases heavily relied upon in the original Interim Guidelines, and cases dealing with a variety of arts are relied upon.

(2) *Comment:* The comments were equally divided with respect to the issue of whether the Guidelines should be broadly applicable to all technologies or limited to biotechnology, DNA claims, or unpredictable arts. Two of the comments urging broad applicability stated that the law should be articulated in a clear and technology neutral fashion, and several comments urged that examples and training materials should illustrate application of the Guidelines in a diverse range of technologies. One comment suggested that applications in which written description problems are likely to arise should be identified generically, rather than requiring a written description analysis in each application. *Response:* The suggestion to cover all technologies and to articulate the law in a clear and technology neutral fashion has been adopted. While a written description analysis is required in each case, the Revised Interim Guidelines clearly specify when a written description issue is most likely to arise, and—for most applications—the Revised Interim Guidelines will quickly lead the examiner to determine that, at least for original claims, the written description requirement has been met. The Revised Interim Guidelines avoid narrowing the application of the written description requirement to a single art, and the examiner training materials will illustrate application of the revision in various technologies.

(3) *Comment:* While the majority of comments supported the Interim Guidelines, eight comments opposed their issuance. Some of those opposing the guidelines argued that the decision in *Regents of the University of California* v. *Eli Lilly*, 119 F.3d 1559, 43 USPQ2d 1398 (Fed. Cir. 1997), *cert. denied*, 523 U.S. 1089 (1998), is a drastic departure from legal precedent and PTO practice. In particular, two comments suggested that the Interim Guidelines should be replaced by Revised Interim Guidelines, and one comment recommended that final Guidelines be deferred until the U.S. Court of Appeals for the Federal Circuit or the U.S. Supreme Court hands down decisions that elaborate, construe, modify, or overrule *Eli Lilly* and/or decide related issues not dealt with by that case. See Comments (5) and (9) for more opposing comments. *Response:* This revision is based on the Office's current understanding of the law and is believed to be fully consistent with binding precedent of the U.S. Supreme Court and the U.S. Court of Appeals for the Federal Circuit. Guidelines are necessary in this area to promote uniformity and consistency in the examination process. The suggestion to issue Revised Interim Guidelines for a second round of Notice and Comment has been adopted. The revision is written in a technology neutral manner, and the form is sufficiently different from the previous guidelines that additional public comment is desired.

(4) *Comment:* Six comments were in favor of including process and product-by-process claims in the analysis, whereas one comment was opposed. One comment criticized the Guidelines for failing to acknowledge the "safe harbor" product-by-process type claim noted in *Fiers* v. *Revel*, 984 F.2d 1164, 25 USPQ2d 1601 (Fed. Cir. 1993), and *Amgen Inc.* v. *Chugai Pharmaceutical Co.*, 927 F.2d 1200, 18 USPQ2d 1016 (Fed. Cir. 1991). One comment observed that process and product-by-process claims tend not to implicate many written description issues, and it may be useful to point out possible enablement deficiencies for such claims. Two comments suggested that the Guidelines should distinguish between claims to processes whose patentability depends on the compositions used in them, as opposed to those where patentability rests in the steps of the process itself. *Response:* The suggestion to address process and product-by-process claims has been adopted. Furthermore, the training materials will analyze claims wherein the patentability depends on the compositions used therein, as well as those where the patentability rests in the process steps themselves. Enablement issues raised by process and product-by-process claims are outside the scope of these Revised Interim Guidelines.

(5) *Comment:* While one comment stated that the Guidelines correctly present the relationship between written description and enablement, a number of comments dispute that the statute actually has a written description requirement distinct from the enablement requirement. One comment requested that the PTO refrain from issuing any Guidelines in this area until the U.S. Supreme Court rules on the Federal Circuit's present position on written description. Several comments urged the PTO to announce that it will not follow the court decisions applying the separate written description requirement, while others observed that the PTO and the practitioners must nevertheless follow the case law. Some of these comments urged the PTO to

withdraw the Guidelines on the grounds that they are premature because the case law has not developed sufficiently. Others urged the PTO to limit application of the Guidelines to the narrow subject matter of the *Fiers, Amgen,* and *Eli Lilly* cases. *Response:* A separate written description requirement has long been a part of the U.S. patent law. *See, e.g., In re Ruschig,* 379 F.2d 990, 154 USPQ 118 (CCPA 1967). The Federal Circuit has recognized the distinct and separable nature of this requirement. *See Vas-Cath.* Although the interpretation of the law is always evolving, the PTO is obliged to follow the law as currently interpreted by the court. As noted above, the suggestion to limit the application of the Revised Interim Guidelines to certain subject matter has not been adopted.

(6) *Comment:* While several of the comments stated that the Guideline's explanation of the purpose of the written description requirement is accurate, a number of comments suggested that the concept of "possession" should be more fully explained or developed. One comment urged that the meaning of "possession of the invention" is different for written description than enablement, whereas another observed that an "in possession of the invention" test for compliance with the written description requirement does not appear in 35 U.S.C. 112, and its definition and application are not clearly stated in the Federal Circuit cases to date. Another comment urged that descriptive attributes which provide proof of written description should include evidence typically provided to prove a complete and enabling conception. One comment stated that the meaning of "has invented" is unclear and queried if actual reduction to practice is required. The same comment asked for clarification on what kind of description equates with possession of a claimed species. One comment stated that a question left unanswered in the Guidelines is that if one has "made" an invention, is one necessarily in possession of it, or are there some further criteria? Two comments observed that physical possession is not necessary: one must have complete conception of the invention in mind. These comments suggested that the possession analysis incorporate the Supreme Court's statements in *Pfaff* v. *Wells Electronics, Inc.,* 525 U.S. 55, 48 USPQ2d 1641 (1998) (the word "invention" must refer to a concept that is complete: one can prove that an invention is complete and ready for patenting before it has been reduced to practice). One of these comments elaborated that the doctrine of simultaneous conception and reduction to practice should remain applicable to only a very small number of cases, including biotechnology cases. *Response:* The Revised Interim Guidelines expand the explanation of possession by discussing decisions that offer some guidance as to how possession may be shown. The concepts in *Pfaff* v. *Wells Electronics* that are pertinent to an analysis of compliance with the written description requirement have been incorporated in this revision. At this time, the Federal Circuit has not indicated that reduction to practice is necessary for conception or written description of a biotechnological invention. The Office does not intend to impose a written description requirement that is more robust than that set forth by the courts. Accordingly, the Revised Interim Guidelines do not impose a *per se* requirement for reduction to practice in any technology to satisfy the written description requirement. However, the Federal Circuit has recognized that in some instances an inventor may only be able to establish a conception (and therefore possession) by pointing to a reduction to practice through a successful experiment. *See Amgen Inc.* v. *Chugai Pharmaceutical Co.,* 927 F.2d at 1206, 18 USPQ2d at 1021. In such instances, the alleged conception fails not merely because the field is unpredictable or because of the general uncertainty surrounding experimental sciences, but because the conception is incomplete due to factual uncertainty that undermines the specificity of the inventor's idea of the invention. *Burroughs Wellcome Co.* v. *Barr Laboratories Inc.,* 40 F.3d 1223, 1229, 32 USPQ2d 1915, 1920 (Fed. Cir. 1994). Reduction to practice in effect provides the only evidence to corroborate conception (and therefore possession) of the invention. *Id.*

(7) *Comment:* Other comments on "possession" urged that possession is to be evaluated by looking to the claims; that the possession question is to be assessed as set forth in *In re Alton,* 76 F.3d 1168, 1176, 37 USPQ2d 1578, 1584 (Fed. Cir. 1996); and that compliance must be assessed on a case-by-case basis given that the question of compliance with the written description requirement is one of fact. One comment stated that the test should be whether the inventor had envisioned the embodiments, not that one of skill in the art can now envision the embodiments. Another comment stated that the Guidelines should take a position with regard to their application to the analysis of declarations submitted under 37 CFR 1.131. *Response:* The Revised Interim Guidelines require the examiner to determine whether there is sufficient written description to inform a skilled artisan that the applicant was in possession of the claimed invention as a whole at the time the application was filed. The revision also indicates that compliance with the written description requirement is a question of fact which must be resolved on a case-by-case basis. While this revision addresses the analysis of possession only in the context of the written description requirement, similar principles apply in determining whether an inventor has met his or her burden of demonstrating possession of the claimed invention in an affidavit or declaration submitted under 37 CFR 1.131.

(8) *Comment:* Several comments suggested that the Guidelines should address questions of support for claims added or amended by the applicant during prosecution (or during an interference). Two comments suggested that the Guidelines should address the "omitted element" prong of the written description requirement. One comment indicated the Guidelines should harmonize chemical and nonchemical case law on when an applicant may amend to broaden or change a definition based on an original disclosure. Another comment stated that the Guidelines should acknowledge that it is proper to amend the claims to excise prior art. *Response:* The suggestions to address questions of support for new or amended claims and to address the "omitted element" test have been adopted.

(9) *Comment:* Several comments indicated that case law such as *In re Koller,* 613 F.2d 819, 204 USPQ 702 (CCPA 1980), hold that original claims constitute their own written description, or that a statement in *ipsis verbis* is a sufficient description, and that those cases should be adhered to. Three comments pointed out that the Guidelines fail to distinguish between original claims and added/amended claims, arguing that the original claim doctrine should exempt originally filed claims from further requirements. *Response:* The Revised Interim Guidelines emphasize that a description as filed is presumed to be adequate, unless or until the examiner introduces sufficient evidence or technical reasoning to the contrary. The original claim doctrine continues to be viable, but the court has indicated that every claim must be supported by sufficient

evidence of possession, and that, under certain circumstances, claim language may not provide an adequate written description of itself. There are no *per se* rules, since the analysis must be done on a case-by-case basis. While original claims have an initial presumption of descriptive support, the applicant should show support for new or amended claims. *See, e.g.*, Manual of Patent Examining Procedure (MPEP) §§ 714.02 and 2163.06 (7th Ed., July 1998) ("Applicant should * * * specifically point out the support for any amendments made to the disclosure.").

(10) *Comment:* One comment indicated that written description problems may arise where there is an inadequate description or demonstration of possession of a genus or where there is an improper genus (no common structure and function that is linked to the practical utility disclosed by the specification). Another comment stated that the Guidelines should address the informational nature of nucleic acid sequences and amino acid sequences. One comment urged that "[a] written description of a genus is sufficient when it is described in enough detail that possession is understood," and that the number of species relates more to enablement. *Response:* The Revised Interim Guidelines indicate that the written description requirement for a claimed genus may be satisfied through sufficient description of a representative number of species. The revision does not require a particular number of species to support a genus, but rather requires that the species adequately described be representative of the claimed genus.

(11) *Comment:* A comment urged that the Guidelines should explicitly state that the maturation of the technology will increase the understanding of one skilled in the art, and ease the predictable scope of the claimed invention beyond the exemplified embodiments, as recognized in the applicant's specification. *Response:* The Revised Interim Guidelines emphasize that in a mature art with a high level of knowledge and skill, less evidence of possession is required.

(12) *Comment:* One comment objected to the requirement for an assessment of predictability as a touchstone for written description. The comment described this inquiry as new and lacking case law support. Several comments stated that predictability is an inquiry relating to the enablement requirement, but not to the written description requirement. Others commented generally that the Guidelines conflate what should be separate enablement and written description analyses. On the other hand, at least one comment stated that the distinctions between these elements converge when lack of enablement results from undue breadth of claims. One comment stated that a review of the application is insufficient to establish the level of predictability in an art. Another queried if the review is to be done after a search in the art and assessment of the art. Another comment stated that the lack of guidance for distinguishing between predictable and unpredictable areas within the field of biotechnology leads to confusion. *Response:* The Revised Interim Guidelines reduce the emphasis on predictability because of the confusion with enablement. Instead, the Guidelines emphasize the knowledge in the art and the skill of the practitioner considered in the totality of the circumstances. With respect to the comment regarding biotechnology, this sliding scale will permit broader claims as the knowledge and skill in this art improve. The Guidelines discuss how the general knowledge in the art may be relied on as evidence of how much description may be needed in particular cases.

(13) *Comment:* Several comments criticized the methodology of the Guidelines because the analytic steps set out by the court in *In re Moore*, 439 F.2d 1232, 169 USPQ 236 (CCPA 1971) (first determine what the claims cover, then review the specification for support) were reversed. *Response:* The Revised Interim Guidelines restate the analytic sequence so it is clearly consistent with *In re Moore*. The revision also makes it clear that each claim must be separately analyzed and given its broadest reasonable interpretation in light of and consistent with the written description. *See, e.g., In re Morris*, 127 F.3d 1048, 1054, 44 USPQ2d 1023, 1027 (Fed. Cir. 1997).

(14) *Comment:* One comment suggested that the Guidelines should provide more instruction on the different amount of description needed to support an essential feature of an invention in contrast to a nonessential feature. The comment explained that contrasting the amount of description needed to support a novel or nonobvious feature of an invention with the amount of description needed for features of an invention that were known in the prior art would be helpful. *Response:* The Revised Interim Guidelines distinguish between novel and old elements in a claim to clarify that the amount of written support needed in an application can vary depending on the general knowledge that was readily available in a particular art.

(15) *Comment:* One comment criticized the analysis for setting out conclusions before the analytic method and for distorting or bypassing the analysis. The same comment said that some of the examples yield illogical results. *Response:* The examples have been deleted from the Guidelines, and the analytical method has been clarified.

(16) *Comment:* The Guidelines were heavily criticized in ten comments for overemphasizing the importance of the preamble and for indicating that generic preamble terms such as "nucleic acid" would need less descriptive support than narrower terms such as "cDNA." One comment objected to the proposition that one may have an adequate written description of a genus of DNA when one does not disclose what gene product the DNA encodes and what that gene product does. This comment recommended deletion of the example bridging F.R. 32640–41 ("a gene comprising SEQ ID NO: 1") as inconsistent with the rest of the Guidelines. *Response:* The Revised Interim Guidelines clarify that the examiner must consider the claim as a whole and that the preamble may be a limitation of the claim. Preamble language is discussed in the context of determining what the claim as a whole encompasses within its scope. However, the Revised Interim Guidelines maintain that any term may trigger a need for more descriptive support because of usage or context. The revision clarifies that during examination claim terms are given their broadest reasonable interpretation consistent with the specification. *See In re Morris*, 127 F.3d 1048, 44 USPQ2d 1023 (Fed. Cir. 1997). The examples have been removed from the text of the revision.

(17) *Comment:* Four comments objected to the Guidelines' definitions for the terms gene, mRNA, and cDNA, stating that the art often refers only to the coding portion of the molecules and does not necessarily imply the presence of regulatory elements or recite specific structures. One comment further indicated that adoption of the PTO's new definition of these terms for purposes of written description considerations could potentially destabilize the economic infrastructure of the biotechnology community because innumerable patents have issued claiming such molecules without regard to the PTO's new interpretation of claim language. The Guidelines were said to use two inconsistent meanings for the term gene that differed in scope and confused the distinction between

genus and species. *Response:* The Revised Interim Guidelines no longer define the term "gene."

(18) *Comment:* One comment indicated that the PTO has the opportunity to emphasize the written description requirement as an anti-submarine patent device; this comment and another observed that two parties could obtain claims which would be almost identical in scope in hindsight, based on completely different paths to the claim. *Response:* In *Hyatt* v. *Boone*, 146 F.3d 1348, 1353, 47 USPQ2d 1128, 1131 (Fed. Cir. 1998), the Federal Circuit addressed the submarine patent issue in finding that the appellant's parent application lacked written descriptive support for a later added claim. When an explicit limitation in a claim "is not present in the written description whose benefit is sought it must be shown that a person of ordinary skill would have understood, at the time the patent application was filed, that the description requires that limitation." *Id.*

(19) *Comment:* A comment stated that the Guidelines give too much emphasis to claim structure, as if the claim is the sole source of the written description. Another comment had a different view, stating that the Guidelines fail to focus on the invention being claimed, and noting that in some circumstances, failure to provide the structure of a gene, enzyme, etc. should not result in finding that a claim containing it fails to meet the written description requirement. *Response:* The Office gives a claim its broadest reasonable interpretation during examination. If the claim taken as a whole requires a limitation not set forth in the original disclosure it may raise an issue of lack of proper written description. As noted in *In re Hiniker Co.*, 150 F.3d 1362, 1369, 47 USPQ2d 1523, 1529 (Fed. Cir. 1998), "the name of the game is the claim."

(20) *Comment:* One comment indicated that there was not enough emphasis on transitional phrases and their impact on the adequacy of the written description. *Response:* As with the preamble, the transitional phrase is discussed in the context of the scope of the claimed invention as a whole.

(21) *Comment:* The Extension of Comment Period and Notice of Hearing requested comments as to how the transition terms "having" and "consisting essentially of" should be treated within the context of nucleotide and amino acid sequence claims. Two comments observed that transitional phrases in the context of nucleotide and amino acid sequence claims should have the same treatment as in chemical cases. Another comment stated that "consisting essentially of" language in DNA or vector claims should not be rejected as *per se* improper under 35 U.S.C. 112, ¶ 2. Two comments stated that lacking an art-accepted meaning or a definition in the specification, "having" would imply an open claim format; another comment stated that "having" is understood to mean "comprising." The term "consisting essentially of" was defined by one comment as a closed claim format that is essentially limited to the compound or composition defined explicitly following the transitional phrase, and by two other comments as having the stated sequence and excluding any alterations which materially change the structure and/or function of the specified sequence. One comment opined that "A DNA consisting essentially of SEQ ID NO: 1" would be limited to DNAs having the nucleotide sequence set forth in SEQ ID NO: 1 plus minor additions at the 5'— and/or 3' —ends of the recited sequence. Another comment observed that the meaning of "consisting essentially of" depends on how the specification defines its usage. *Response:* During examination, the claim as a whole is given the broadest reasonable interpretation consistent with the specification. Transitional phrases should be given the same treatment in all cases. The Revised Interim Guidelines set forth legally recognized definitions for transition language in an endnote. "Consisting essentially of" is acceptable transition language in nucleic acid and protein claims. The impact of the transition language on enablement and practical utility will not be dealt with in this forum.

(22) *Comment:* One comment criticized the use of the taxonomic terms "genus" and "species." The comment explained that because the terminology is well established in biology, it should not be applied to chemical compounds. Two comments described the Guidelines as deficient in analyzing the proper relationship of preamble, transitional phrase and claim body for distinguishing genus from species claims. According to another comment, the Guidelines confuse genus and species claims. *Response:* The Revised Interim Guidelines refer to the terms "genus" and "species" in their well accepted legal sense as widely used patent terms of art that are recognized as distinct from their use as taxonomic terms. The revision clarifies what is meant by genus and species.

(23) *Comment:* Several comments found the explanations for the examples deficient because they do not clarify what would constitute a sufficient disclosure. One comment urged that there is no guidance provided as to what would constitute sufficient identifying characteristics, and the Guidelines do not set forth the number of the examples needed for sufficient written description. Another comment urged that structure, or function plus partial structure, or function plus "some characteristics" (*e.g.*, 2 or more), is sufficient to meet the written description requirement. Yet another comment urged that uncertainties and potential problems exist because it is unclear how "relevant" or "sufficient" identifying characteristics are established; that it is unclear how functional properties fit into the analysis; and that problems exist with the level of uncertainty when the complete structure is not disclosed or the structure is not disclosed and only a few identifying characteristics are disclosed. Another comment urged that the methodology is incomplete as to how many identifying characteristics are required and what characteristics are relevant for description of a species. This comment applied the same reasoning to the number of species required for describing a genus. One comment urged that functional characteristics in combination with certain objectively defined physical characteristics can serve to characterize the compound sufficiently to establish possession, even in less developed arts. One comment urged that the ability to predict structure from function is given as a standard for the written description requirement without any citation to authority. *Response:* The Revised Interim Guidelines do not include examples within the text. The test for whether sufficient identifying characteristics have been disclosed is not a bright-line test, but rather requires weighing various factors including the level of skill and knowledge in the art, and the extent to which relevant identifying characteristics are described. The revision provides more guidance to the examiners by citing as examples cases involving mature arts with a high level of skill and knowledge (*e.g.*, *Pfaff* v. *Wells Electronics, Fonar Corp.* v. *General Electric Co.*, 107 F.3d 1543, 1549, 41 USPQ2d 1801, 1805 (Fed. Cir. 1997) and *Vas-Cath* v. *Mahurkar*), as well as cases in emerging technologies where more description is necessary (*e.g.*, *Eli Lilly, Amgen* v. *Chugai*, and *Fiers* v. *Revel*). The test remains whether one of skill in the art, provided with the disclosure, would recognize that the applicant was in possession of the claimed subject matter when the application was filed.

(24) *Comment:* The Extension of Comment Period and Notice of Hearing requested comments on how the final Guidelines should address the deposit of a biological material made under 37 CFR 1.801, and comments on the extent to which a deposit of biological material may be relied upon to support the addition or correction of sequence information. Several comments expressed the opinion that deposit of a compound or biological material can be one means of demonstrating possession of a specifically claimed compound that has not otherwise been described in a complete manner in the specification. One comment stated that if a gene were cloned but not sequenced, and the vector in question were deposited, the sequence is an inherent property of the deposited vector and hence the description requirement would be satisfied if the claim referred to the deposit. One comment urged that the description requirement may be satisfied by the inherent properties of a disclosed structure, citing *Kennecott Corp.* v. *Kyocera Int'l Inc.*, 835 F.2d 1419, 5 USPQ2d 1194 (Fed. Cir. 1987). As for the later addition or correction of information, several comments indicated that actual possession established through a deposit with a partial characterization (*i.e.*, to correlate the physical description to the material that has been deposited, such as molecular weight, partial sequence) should be sufficient to avoid problems with new matter where the information added to a disclosure is an inherent characteristic of the compound or composition. One comment indicated that correcting a sequence based on more accurate sequencing of deposited material does not introduce new matter. One comment stated that present genus-species concepts should prevent an applicant from obtaining an unfair advantage by depositing a large amount of material and then relying on inherency; if a variety of materials are deposited in a single host, the specification must adequately describe how to isolate the intended molecule(s). Two comments expressly stated "no comment" with regard to the issue of adding a substantial amount of sequence information. One comment opined that the date of deposit is not controlling with regard to the issue of whether the written description requirement is met, and a second comment observed that *In re Lundak*, 773 F.2d 1216, 227 USPQ 90 (Fed. Cir. 1985), cannot be limited by rule. *Response:* The Revised Interim Guidelines indicate that a deposit of a claimed biological material in accordance with the requirements of 37 CFR 1.801 *et seq.* is evidence of actual reduction to practice of the biological material. However, a deposit is not a substitute for a written description of the claimed invention. The Revised Interim Guidelines also address the issue of when a deposit can be relied upon to correct minor sequencing errors. However, addition of sequence information based on a deposit is not specifically addressed; these circumstances create issues yet to be resolved by the courts, and will be resolved on a case-by-case basis in the PTO. *See, e.g., In re Fisher*, 427 F.2d 833, 836, 166 USPQ 18, 21 (CCPA 1970).

(25) *Comment:* One comment explained that associating taxonomic groupings with gene sequences is a dated concept because genes are not distinguishable as to origin. The generic term "mammal gene" was said to be meaningless, absent an implied process limitation that the gene was obtained from a mammal. *Response:* The examples have been removed from the revision. However, the training materials will permit applicants to use taxonomic modifiers such as "mammalian" because the usage is ubiquitous in the literature and in patents and generally has an accepted meaning in the art.

(26) *Comment:* One comment urged that broad functional claims lacking defining structure should not be granted on the basis of a "not easily generalizable disclosure." A different comment stated that functional characteristics can be appropriate in all arts. Comments differed on hybridization, where some held it is a proper defining characteristic, and another stated it is insufficient. *Response:* The Revised Interim Guidelines do not establish per se rules regarding functional language. When used appropriately, functional language may provide an adequate written description of the claims invention as discussed in the Revised Interim Guidelines.

(27) *Comment:* Several comments indicated that the Guidelines present inadequate guidance with respect to analyzing written description support for genus claims. One comment stated that the Guidelines provide inadequate criteria for selection of appropriate genuses. Another comment stated that the Guidelines do not provide adequate guidance to determine whether an applicant has presented a properly formed genus, and suggested that "a genus designation should be strictly tied to the disclosed properties of the structures being claimed." Another comment stated that the Guidelines should clarify that the genus/species distinction is determined by the transitional phrase and body of the claim, not the preamble. Another comment stated that the Guidelines provide inadequate guidance as to the number of species required to meet the written description requirement for a genus. One comment urged that a relevant factor to consider is whether the claims cover embodiments broader than the essential elements of the embodiments described in the specification as in *Gentry Gallery Inc.* v. *Berkline*, 134 F.3d 1473, 45 USPQ2d 1498 (Fed. Cir. 1998). According to this comment, species rarely, if ever, constitute sufficient support for generic claims unless accompanied by a general disclosure that is commensurate in scope with the claims. *Response:* The Revised Interim Guidelines follow Federal Circuit case law which requires a representative number of species to satisfy the written description requirement for a genus. Written description is a question of fact, and what constitutes a representative number for a genus is a factual determination left to a case-by-case analysis by the examiner.

(28) *Comment:* One comment urged that general allegations of "unpredictability in the art" are insufficient to support a case against the applicant, and that examiners should be instructed to weigh applicant's evidence of what the description provides to one of skill in the art. *Response:* The suggestion to clarify that a general allegation of "unpredictability in the art" is insufficient to support a rejection has been adopted. A disclosure as filed is *prima facie* adequate. To support a rejection, the PTO has the burden of showing why the applicant's evidence is insufficient. In any case where lack of written description is found, the PTO should cite documentary evidence in support of the finding. Where documentary evidence is not available, technical reasoning, as distinguished from legal reasoning, may support the finding when the technical line of reasoning relates to fact finding regarding possession of the invention.

(29) *Comment:* One comment indicated that rejections based on the enablement and written description requirements of 35 U.S.C. 112 should be made separately, and the rejections should not mix standards. *Response:* Examiners are directed to make separate rejections based on the enablement and written description requirements of 35 U.S.C. 112. *See, e.g.*, MPEP § 706.03(c) (explaining when it is appropriate to use a particular form paragraph for rejecting claims under 35 U.S.C. 112, ¶1) and MPEP § 2164 ("limitations must be

analyzed for both enablement and description using their separate and distinct criteria").

(30) *Comment*: One comment observed that the Guidelines do not guide examiners in how to suggest amendments to bring the claims into compliance. The comment also observed that examiners may be ill-equipped to deal with evaluating the sufficiency of applicant's efforts. *Response:* The training materials will provide guidance as to how rejections for lack of an adequate written description can be overcome.

(31) *Comment*: One comment stated that the Guidelines should instruct examiners to pay due regard to the scientific and commercial realities of each individual invention, such that the scope of the claims is a fair reflection of the applicant's contribution to the art. *Response*: The scientific and commercial realities of each invention are considered to the extent that they impact analysis of a claimed invention for compliance with Title 35 of the U.S. Code. The Office is bound to follow the law and cannot make judgment calls as to what is "a fair reflection of the applicant's contribution to the art."

(32) *Comment:* While two comments observed that the Guidelines should not have a significant impact on patents or pending or newly filed applications because they are only Guidelines which are not binding on the Board or examiners, three comments were of the opinion that the Guidelines would impact pending and newly filed cases by limiting the scope of patent protection. One comment was of the opinion that the Guidelines should have no impact on issued cases except reissues, whereas another expected many issued patents to be declared invalid (more as a result of *Eli Lilly* than the Guidelines). Another comment observed that the Guidelines should not impose significant new burdens on patent applicants in the biotechnology arts or give rise to a new "anti-patenting" posture in the biotechnology examination group; however, the PTO should not be misled into adapting "customer-friendly" examination standards that do not subject applications to a thorough and rigorous examination. One comment opined that the Guidelines will result in a great increase in the number of appeals until the Federal Circuit makes clear that the law is quite different, thus delaying commercialization of potentially life improving and life saving inventions. According to this comment, universities and small inventors do not have the financial support to provide the exhaustive kind of work the Guidelines can require for meaningful coverage; this will mean that many biotechnology inventions will not be commercialized. One comment stated that the Commissioner indicates that meaningful patent coverage is required for commercial exploitation of biotechnological inventions, yet the PTO continues to take a position that leads away from what the Commissioner espouses. Another comment felt that the scope of allowed claims would be dependent on the examiner; a potential applicant would not know what sort of claims could be obtained based on a particular disclosure. One comment opined that applications filed after publication of the Guidelines will probably be much more detailed and longer in length. *Response*: The Revised Interim Guidelines clarify that a written description issue should rarely arise for an original claim because such a claim is presumed to have adequate descriptive support. The burden is on the examiner to provide evidence or reasoning in support of any rejection. Such an approach would not be expected to increase the number of appeals, nor should it require exhaustive work for meaningful coverage. The Revised Interim Guidelines are intended to promote uniformity, not diminish it.

(33) *Comment*: One comment indicated it is premature to instruct examiners in the proposed Guidelines since they may change dramatically as a result of public comment. Three comments stated that the Guidelines should not be applied until final Guidelines have been approved; two of these indicated that the Guidelines should only be applied to applications filed after implementation. One comment suggested preparing separate guidance for currently pending applications. *Response*: Separate guidance is not required for pending applications and applications filed after implementation of any final Guidelines; the Guidelines do not establish new law or rules or impose any additional requirements on applicants.

(34) *Comment*: One comment requested that the PTO address the issue of open-claim language for EST claims in the final Guidelines because of their importance to the biotechnology industry. Several comments stated that permitting open-ended language with respect to an EST claim contradicts the written description requirement because the common structural features of the EST do not constitute a "substantial portion of the genus" as required by the *Eli Lilly* case. According to these commentators, a claim such as "a DNA comprising SEQ. ID. NO: 1" would lack written description when SEQ. ID. NO: 1 was a gene fragment. *Response*: The Revised Interim Guidelines maintain the view that use of such terms as "gene" in the preamble of an EST claim may raise a written description issue if one skilled in the art would understand that a "gene" requires elements which are not sufficiently described. However, claims to "a DNA comprising SEQ. ID. NO: 1" are unlikely to raise a written description issue. The comments do not explain why there is a written description problem for a claim such as "a DNA comprising SEQ. ID. 1" when SEQ. ID. 1 is an EST, while there is no problem when SEQ. ID. 1 is a whole gene or a gene promoter. The only difference seems to be the utility of the DNA fragment.

(35) *Comment*: One comment asserted that the scope and level of unpredictability of the structure is so large that the person skilled in the art could not envisage sufficient species to place the genus in possession of the inventor at the time of filing, and that it should be a rare disclosure that supports EST claims broader than the specific SEQ. ID, even for claims such as "a DNA comprising the EST of SEQ. ID. NO: 1." The comment also suggested that claim language that supports the introduction of an infinite amount of random sequence would require an immense number of exemplary species. Several commentators advanced the position that disclosure of only a small fragment does not convey that the inventor was in possession of all of the possible molecules or that the inventor was in possession of the fragment wherever it occurs. *Response*: A claim such as "a DNA comprising the EST of SEQ. ID. NO: 1" or "a gene comprising the EST of SEQ. ID. NO: 1" will be analyzed for compliance with the written description requirement by determining whether the partial structure in combination with any other disclosed relevant identifying characteristics are sufficient to show that a skilled artisan would recognize that the applicant was in possession of the claimed invention as a whole. The Office does not agree with the comment that the scope of such an EST claim is necessarily too large to satisfy the written description requirement. The PTO has issued numerous patents in the past directed to nucleic acids that use open-ended language. Although an applicant presenting an original claim to an EST using open-ended claim language with disclosure of only the EST sequence is not in possession of any arbitrary specific possible molecule that contains the EST, the applicant may

be in possession of a broad genus of DNA where the EST is in any random nucleic acid sequence. The comment's statement to the contrary would preclude open-ended claims incorporating any DNA sequence such as gene or promoter. In fact, such a view would appear to preclude open-ended language for any other polymer. However, such open-ended EST claims may not comply with the utility and scope of enablement requirements of 35 U.S.C. 101 and 112.

Revised Interim Guidelines for the Examination of Patent Applications Under the 35 U.S.C. § 112, ¶ 1 "Written Description" Requirement

These revised interim "Written Description Guidelines" are intended to assist Office personnel in the examination of patent applications for compliance with the written description requirement of 35 U.S.C. 112, ¶ 1. This revision is based on the Office's current understanding of the law and public comments received in response to the PTO's previous request for public comments on its Interim Written Description Guidelines and is believed to be fully consistent with binding precedent of the U.S. Supreme Court, as well as the U.S. Court of Appeals for the Federal Circuit and its predecessor courts.

This revision does not constitute substantive rulemaking and hence does not have the force and effect of law. It is designed to assist Office personnel in analyzing claimed subject matter for compliance with substantive law. Rejections will be based upon the substantive law, and it is these rejections which are appealable. Consequently, any perceived failure by Office personnel to follow the Revised Interim Guidelines is neither appealable nor petitionable.

These Revised Interim Guidelines are intended to form part of the normal examination process. Thus, where Office personnel establish a *prima facie* case of lack of written description for a claim, a thorough review of the prior art and examination on the merits for compliance with the other statutory requirements, including those of 35 U.S.C. 101, 102, 103, and 112, is to be conducted prior to completing an Office action which includes a rejection for lack of written description. Office personnel are to rely on this revision of the guidelines in the event of any inconsistent treatment of issues involving the written description requirement between these Revised Interim Guidelines and any earlier guidance provided from the Office.

I. General Principles Governing Compliance With the "Written Description" Requirement for Applications

The first paragraph of 35 U.S.C. 112 requires that the "specification shall contain a written description of the invention. * * *" This requirement is separate and distinct from the enablement requirement.[1] The written description requirement has several policy objectives. "[T]he 'essential goal' of the description of the invention requirement is to clearly convey the information that an applicant has invented the subject matter which is claimed."[2] Another objective is to put the public in possession of what the applicant claims as the invention. The written description requirement of the Patent Act promotes the progress of the useful arts by ensuring that patentees adequately describe their inventions in their patent specifications in exchange for the right to exclude others from practicing the invention for the duration of the patent's term.[3]

To satisfy the written description requirement, a patent specification must describe the claimed invention in sufficient detail that one skilled in the art can reasonably conclude that the inventor had possession of the claimed invention.[4] An applicant shows possession of the claimed invention by describing the claimed invention with all of its limitations.[5] Possession may be shown by actual reduction to practice,[6] or by showing that the invention was "ready for patenting" such as by the disclosure of drawings or other descriptions of the invention that are sufficiently specific to enable a person skilled in the art to practice the invention.[7] A question as to whether a specification provides an adequate written description may arise in the context of an original claim which is not described sufficiently, a new or amended claim wherein a claim limitation has been added or removed, or a claim to entitlement of an earlier priority date or effective filing date under 35 U.S.C. 119, 120, or 365(c).[8] Compliance with the written description requirement is a question of fact which must be resolved on a case-by-case basis.[9]

A. Original Claims

There is a strong presumption that an adequate written description of the claimed invention is present when the application is filed.[10] However, the issue of a lack of adequate written description may arise even for an original claim when an aspect of the claimed invention has not been described with sufficient particularity such that one skilled in the art would recognize that the applicant had possession of the claimed invention.[11] The claimed invention as a whole may not be adequately described if the claims require an essential or critical element which is not adequately described in the specification and which is not conventional in the art.[12] This problem may arise where an invention is described solely in terms of a method of its making coupled with its function and there is no described or art recognized correlation or relationship between the structure of the invention and its function.[13] A lack of adequate written description problem also arises if the knowledge and level of skill in the art would not permit one skilled in the art to immediately envisage the product claimed from the disclosed process.[14]

B. New or Amended Claims

The proscription against the introduction of new matter in a patent application[15] serves to prevent an applicant from adding information that goes beyond the subject matter originally filed.[16] Thus, the written description requirement prevents an applicant from claiming subject matter that was not adequately described in the specification as filed. New or amended claims which introduce elements or limitations which are not supported by the as-filed disclosure violate the written description requirement.[17] While there is no *in haec verba* requirement, newly added claim limitations must be supported in the specification through express, implicit, or inherent disclosure. An amendment to correct an obvious error does not constitute new matter where one skilled in the art would not only recognize the existence of the error in the specification, but also the appropriate correction.[18]

Under certain circumstances, omission of a limitation can raise an issue regarding whether the inventor had possession of a broader, more generic invention.[19] A claim that omits an element which applicant describes as an essential or critical feature of the invention originally disclosed does not comply with the written description requirement.[20]

The fundamental factual inquiry is whether the specification conveys with reasonable clarity to those skilled in the art that, as of the filing date sought, applicant was in possession of the invention as now claimed.[21]

[1] See Endnotes at end of this notice.

II. Methodology for Determining Adequacy of Written Description

A. Read and Analyze the Specification for Compliance With 35 U.S.C. 112, ¶ 1

Office personnel should adhere to the following procedures when reviewing patent applications for compliance with the written description requirement of 35 U.S.C. 112, ¶ 1. The examiner has the initial burden, after a thorough reading and evaluation of the content of the application, of presenting evidence or reasons why a person skilled in the art would not recognize that the written description of the invention provides support for the claims. There is a strong presumption that an adequate written description of the claimed invention is present in the specification as filed;[22] however, with respect to newly added or amended claims, applicant should show support in the original disclosure for the new or amended claims.[23] Consequently, rejection of an original claim for lack of written description should be rare. The inquiry into whether the description requirement is met is a question of fact that must be determined on a case-by-case basis.[24]

1. For Each Claim, Determine What the Claim as a Whole Covers

Claim construction is an essential part of the examination process. Each claim must be separately analyzed and given its broadest reasonable interpretation in light of and consistent with the written description.[25] The entire claim must be considered, including the preamble language[26] and the transitional phrase.[27] The claim as a whole, including all limitations found in the preamble,[28] the transitional phrase, and the body of the claim, must be sufficiently described in the specification to satisfy the written description requirement.[29]

The examiner should evaluate each claim to determine if sufficient structures, acts, or functions are recited to make clear the scope and meaning of the claim, including the weight to be given the preamble.[30] The absence of definitions or details for well-established terms or procedures should not be the basis of a rejection under 35 U.S.C. 112, ¶ 1, for lack of adequate written description. Limitations may not, however, be imported into the claims from the specification.

2. Review the Entire Application to Understand What Applicant Has Described as the Essential Features of the Invention

Prior to determining whether the disclosure satisfies the written description requirement for the claimed subject matter, the examiner should review the claims and the entire specification, including the specific embodiments, figures, and sequence listings, to understand what applicant has identified as the essential distinguishing characteristics of the invention. The analysis of whether the specification complies with the written description requirement requires the examiner to determine the correspondence between what applicant has described as the essential identifying characteristic features of the invention, *i.e.*, what the applicant has demonstrated possession of, and what applicant has claimed. Such a review is conducted from the standpoint of one of skill in the art at the time the application was filed,[31] and should include a determination of the field of the invention and the level of skill and knowledge in the art. Generally, there is an inverse correlation between the level of skill and knowledge in the art and the specificity of disclosure necessary to satisfy the written description requirement. Information which is well known in the art does not have to be described in detail in the specification.[32]

3. Determine Whether There is Sufficient Written Description To Inform a Skilled Artisan That Applicant Was in Possession of the Claimed Invention as a Whole at the Time the Application Was Filed

a. Original claims.—Possession may be shown in any number of ways. Possession may be shown by actual reduction to practice, by a clear depiction of the invention in detailed drawings which permit a person skilled in the art to clearly recognize that applicant had possession of the claimed invention, or by a written description of the invention describing sufficient relevant identifying characteristics such that a person skilled in the art would recognize that the inventor had possession of the claimed invention.[33]

A specification may show actual reduction to practice by showing that the inventor constructed an embodiment or performed a process that met all the limitations of the claim, and determined that the invention would work for its intended purpose.[34] Actual reduction to practice of a biological material may be shown by specifically describing a deposit made in accordance with the requirements of 37 C.F.R. § 1.801 *et seq.*[35]

An applicant may show possession of an invention by disclosure of drawings that are sufficiently detailed to show that applicant was in possession of the claimed invention as a whole.[36] The description need only describe in detail that which is new or not conventional.[37] This is equally true whether the claimed invention is directed to a product or a process. Normally a reduction to drawings will adequately describe the claimed invention.[38]

An applicant may also show that an invention is complete by disclosure of sufficiently detailed relevant identifying characteristics which provide evidence that applicant was in possession of the claimed invention.[39] *i.e.*, complete or partial structure, other physical and/or chemical properties, functional characteristics when coupled with a known or disclosed correlation between function and structure, or some combination of such characteristics.[40] What is conventional or well known to one skilled in the art need not be disclosed in detail.[41] If a skilled artisan would have understood the inventor to be in possession of the claimed invention at the time of filing, even if every nuance of the claims is not explicitly described in the specification, then the adequate description requirement is met.[42]

(1) For each claim drawn to a single embodiment or species:[43]

(a) Determine whether the application describes an actual reduction to practice of the claimed invention.

(b) If the application does not describe an actual reduction to practice, determine whether the invention is complete as evidenced by a reduction to drawings.

(c) If the application does not describe an actual reduction to practice or reduction to drawings, determine whether the invention has been set forth in terms of distinguishing identifying characteristics as evidenced by other descriptions of the invention that are sufficiently detailed to show that applicant was in possession of the claimed invention.

(i) Determine whether the application as filed describes the complete structure (or acts of a process) of the claimed invention as a whole. The complete structure of a species or embodiment typically satisfies the requirement that the description be set forth "in such full, clear, concise, and exact terms" to show possession of the claimed invention.[44] If a complete structure is disclosed, the written description requirement is satisfied for that species or embodiment, and a rejection under 35 U.S.C. 112, ¶ 1 for lack of written description must not be made.

(ii) If the application as filed does not disclose the complete structure (or acts of a process) of the claimed invention as a whole, determine whether the specification discloses other relevant identifying characteristics sufficient to

describe the claimed invention in such full, clear, concise, and exact terms that a skilled artisan would recognize applicant was in possession of the claimed invention.[45] Whether the specification shows that applicant was in possession of the claimed invention is not a single, simple factual determination, but rather is a conclusion reached by weighing many factual considerations. Factors to be considered in determining whether there is sufficient evidence of possession include the level of skill and knowledge in the art, partial structure, physical and/or chemical properties, functional characteristics alone or coupled with a known or disclosed correlation between structure and function, and the method of making the claimed invention. Disclosure of any combination of such identifying characteristics that distinguish the claimed invention from other materials and would lead one of skill in the art to the conclusion that the applicant was in possession of the claimed species is sufficient. Patents and printed publications in the art should be relied upon to determine whether an art is mature and what the level of knowledge and skill is in the art. In most technologies which are mature, and wherein the knowledge and level of skill in the art is high, a written description question should not be raised for original claims even if the specification discloses only a method of making the invention and the function of the invention.[46] In contrast, in emerging and unpredictable technologies, more evidence is required to show possession. For example, disclosure of only a method of making the invention and the function may not be sufficient to support a product claim other than a product-by-process claim.[47] Furthermore, disclosure of partial structure without additional characterization of the product may not be sufficient to evidence possession of the claimed invention.[48]

Any claim to a species that does not meet the test described under at least one of (a), (b), or (c) must be rejected as lacking adequate written description under 35 U.S.C. 112, ¶ 1.

(2) For each claim drawn to a genus:

The written description requirement for a claimed genus may be satisfied through sufficient description of a representative number of species by actual reduction practice (see (1)(a), above), reduction to drawings (see (1)(b), above), or by disclosure of relevant identifying characteristics, *i.e.*, structure or other physical and/or chemical properties, by functional characteristics coupled with a known or disclosed correlation between function and structure, or by a combination of such identifying characteristics, sufficient to show the applicant was in possession of the claimed genus (see (1)(c), above).[49]

A "representative number of species" means that the species which are adequately described are representative of the entire genus. Thus, when there is substantial variation within the genus, one must describe a sufficient variety of species to reflect the variation within the genus. What constitutes a "representative number" is an inverse function of the skill and knowledge in the art. Satisfactory disclosure of a "representative number" depends on whether one of skill in the art would recognize that the applicant was in possession of the necessary common attributes or features of the elements possessed by the members of the genus in view of the species disclosed. In an unpredictable art, adequate written description of a genus which embraces widely variant species *cannot* be achieved by disclosing only one species within the genus.[50] Description of a representative number of species does not require the description to be of such specificity that it would provide individual support for each species that the genus embraces.[51] If a representative number of adequately described species are not disclosed for a genus, the claim to that genus must be rejected as lacking adequate written description under 35 U.S.C. 112, ¶ 1.

b. New claims, amended claims, or claims asserting entitlement to the benefit of an earlier priority date or filing date under 35 U.S.C. §§ 119, 120, or 365(c).—The examiner has the initial burden of presenting evidence or reasoning to explain why persons skilled in the art would not recognize in the original disclosure a description of the invention defined by the claims.[52] However, when filing an amendment an applicant should show support in the original disclosure for new or amended claims.[53] To comply with the written description requirement of 35 U.S.C. 112, ¶ 1, or to be entitled to an earlier priority date or filing date under 35 U.S.C. 119, 120, or 365(c), each claim limitation must be expressly,[54] implicitly,[55] or inherently[56] supported in the originally filed disclosure.[57] Furthermore, each claim must include all elements which applicant has described as essential.[58]

If the originally filed disclosure does not provide support for each claim limitation, or if an element which applicant describes as essential or critical is not claimed, a new or amended claim must be rejected under 35 U.S.C. 112, ¶ 1, as lacking adequate written description, or in the case of a claim for priority under 35 U.S.C. 119, 120, or 365(c), the claim for priority must be denied.

III. Complete Patentability Determination Under All Statutory Requirements and Clearly Communicate Findings, Conclusions and Their Bases

The above only describes how to determine whether the written description requirement of 35 U.S.C. 112, ¶ 1 is satisfied. Regardless of the outcome of that determination, Office personnel must complete the patentability determination under all the relevant statutory provisions of Title 35 of the U.S. Code.

Once Office personnel have concluded analysis of the claimed invention under all the statutory provisions, including 35 U.S.C. 101, 112, 102, and 103, they should review all the proposed rejections and their bases to confirm their correctness. Only then should any rejection be imposed in an Office action. The Office action should clearly communicate the findings, conclusions, and reasons which support them. When possible, the Office action should offer helpful suggestions on how to overcome rejections.

A. For Each Claim Lacking Written Description Support, Reject the Claim Under Section 112, ¶1, for Lack of Adequate Written Description

A description as filed is presumed to be adequate, unless or until sufficient evidence or reasoning to the contrary has been presented by the examiner to rebut the presumption.[59] The examiner, therefore, must have a reasonable basis to challenge the adequacy of the written description. The examiner has the initial burden of presenting by a preponderance of evidence why a person skilled in the art would not recognize in an applicant's disclosure a description of the invention defined by the claims.[60] In rejecting a claim, the examiner must set forth express findings of fact regarding the above analysis which support the lack of written description conclusion. These findings should:

(1) identify the claim limitation at issue; and

(2) establish a *prima facie* case by providing reasons why a person skilled in the art at the time the application was filed would not have recognized that the inventor was in possession of the invention as claimed in view of the disclosure of the application as filed. A general allegation of "unpredictability

in the art" is not a sufficient reason to support a rejection for lack of adequate written description.

When appropriate, suggest amendments to the claims which can be supported by the application's written description, being mindful of the prohibition against the addition of new matter in the claims or description.[61]

B. Upon Reply By Applicant, Again Determine the Patentability of the Claimed Invention, Including Whether the Written Description Requirement is Satisfied by Reperforming the Analysis Described Above in View of the Whole Record

Upon reply by applicant, before repeating any rejection under 35 U.S.C. 112, ¶ 1 for lack of written description, review the basis for the rejection in view of the record as a whole, including amendments, arguments, and any evidence submitted by applicant. If the whole record now demonstrates that the written description requirement is satisfied, do *not* repeat the rejection in the next Office action. If the record still does not demonstrate that written description is adequate to support the claim(s), repeat the rejection under 35 U.S.C. 112, ¶ 1, fully respond to applicant's rebuttal arguments, and properly treat any further showings submitted by applicant in the reply. Any affidavits, including those relevant to the 112, ¶ 1, written description requirement,[62] must be thoroughly analyzed and discussed in the next Office action.

ENDNOTES

1. *See, e.g., Vas-Cath, Inc.* v. *Mahurkar*, 935 F.2d 1555, 1560, 19 USPQ2d 1111, 1114 (Fed. Cir. 1991).

2. *In re Barker*, 559 F.2d 588, 592 n.4, 194 USPQ 470, 473 n.4 (CCPA 1977).

3. *See Regents of the University of California* v. *Eli Lilly*, 119 F.3d 1559, 1566, 43 USPQ2d 1398, 1404 (Fed. Cir. 1997), *cert. denied*, 523 U.S. 1089 (1998).

4. *See, e.g., Vas-Cath, Inc.* v. *Mahurkar*, 935 F.2d at 1563, 19 USPQ2d at 1116. Much of the written description case law addresses whether the specification as originally filed supports claims not originally in the application. The issue raised in the cases is most often phrased as whether the original application provides "adequate support" for the claims at issue or whether the material added to the specification incorporates "new matter" in violation of 35 U.S.C. § 132. The "written description" question similarly arises in the interference context, where the issue is whether the specification of one party to the interference can support the newly added claims corresponding to the count at issue, *i.e.*, whether that party can "make the claim" corresponding to the interference count. *E.g., see Martin* v. *Mayer*, 823 F.2d 500, 502, 3 USPQ2d 1333, 1335 (Fed. Cir. 1987).

In addition, early opinions suggest the Patent and Trademark Office was unwilling to find written descriptive support when the only description was found in the claims; however, this viewpoint was rejected. *See In re Koller*, 613 F.2d 819, 204 USPQ 702 (CCPA 1980) (original claims constitute their own description); *In re Gardner*, 475 F.2d 1389, 177 USPQ 396 (CCPA 1973) (accord); *In re Wertheim*, 541 F.2d 257, 191 USPQ 90 (CCPA 1976) (accord). It is now well accepted that a satisfactory description may be in the claims or any other portion of the originally filed specification.

These early opinions did not address the quality or specificity of particularity that was required in the description, *i.e.*, how much description is enough.

5. *Lockwood* v. *American Airlines, Inc.*, 107 F.3d 1565, 1572, 41 USPQ2d 1961, 1966 (Fed. Cir. 1997).

6. An application specification may show actual reduction to practice by describing testing of the claimed invention or, in the case of biological materials, by specifically describing a deposit made in accordance with 37 CFR 1.801 *et seq.* 37 CFR 1.804, 1.809. *See also Deposit of Biological Materials for Patent Purposes, Final Rule*, 54 FR 34,864 (August 22, 1989) ("The requirement for a specific identification is consistent with the description requirement of the first paragraph of 35 U.S.C. 112, and to provide an antecedent basis for the biological material which either has been or will be deposited before the patent is granted." *Id.* at 34876. "[T]he description must be sufficient to permit verification that the deposited biological material is in fact that disclosed. Once the patent issues, the description must be sufficient to aid in the resolution of questions of infringement." *Id.* at 34,880.). Such a deposit is not a substitute for a written description of the claimed invention. The written description of the deposited material needs to be as complete as possible because the examination for patentability proceeds solely on the basis of the written description. *See, e.g., In re Lundak*, 773 F.2d 1216, 227 USPQ 90 (Fed. Cir. 1985). *See also* 54 FR at 34,880 ("As a general rule, the more information that is provided about a particular deposited biological material, the better the examiner will be able to compare the identity and characteristics of the deposited biological material with the prior art.").

7. *Pfaff* v. *Wells Electronics, Inc.*, 525 U.S. 55,___, 119 S.Ct. 304, 312, 48 USPQ2d 1641, 1647 (1998).

8. A description requirement issue can arise for original claims (*see, e.g., Eli Lilly*, 119 F.3d 1559, 43 USPQ2d 1398) as well as new or amended claims. Most typically, the issue will arise in the context of determining whether new or amended claims are supported by the description of the invention in the application as filed (*see, e.g., In re Wright*, 866 F.2d 422, 9 USPQ2d 1649 (Fed. Cir. 1989)), whether a claimed invention is entitled to the benefit of an earlier priority date or effective filing date under 35 U.S.C. 119, 120, or 365(c) (*see, e.g., Tronzo* v. *Biomet, Inc.*, 156 F.3d 1154, 47 USPQ2d 1829 (Fed. Cir. 1998); *Fiers* v. *Revel*, 984 F.2d 1164, 25 USPQ2d 1601 (Fed. Cir. 1993); *In re Ziegler*, 992 F.2d 1197, 1200, 26 USPQ2d 1600, 1603 (Fed. Cir. 1993)), or whether a specification provides support for a claim corresponding to a count in an interference (*see, e.g., Fields* v. *Conover*, 443 F.2d 1386, 170 USPQ 276 (CCPA 1970)).

9. *Vas-Cath, Inc.* v. *Mahurkar*, 935 F.2d at 1563, 19 USPQ2d at 1116 (Fed. Cir. 1991).

10. *In re Wertheim*, 541 F.2d at 262, 191 USPQ at 96.

11. See endnote 4.

12. For example, consider the claim "A gene comprising SEQ ID NO: 1." A determination of what the claim as a whole covers may result in a conclusion that specific structures such as a promoter, a coding region, or other elements are included. Although all genes encompassed by this claim share the characteristic of comprising SEQ ID NO: 1, there may be insufficient description of those specific structures (*e.g.*, promoters, enhancers, coding regions, and other regulatory elements) which are also included.

13. A biomolecule sequence described only by a functional characteristic, without any known or disclosed correlation between that function and the structure of the sequence, normally is not a sufficient identifying characteristic for written description purposes, even when accompanied by a method of obtaining the claimed sequence. For example, even though a genetic code table would correlate a known amino acid sequence with a genus of coding nucleic acids, the same table cannot predict the native, naturally occurring nucleic acid sequence of a naturally occurring mRNA or its corresponding cDNA. *Cf. In re Bell*, 991 F.2d 781, 26 USPQ2d 1529 (Fed. Cir. 1993), and *In re Deuel*, 51 F.3d 1552, 34 USPQ2d 1210 (Fed. Cir. 1995) (holding that a process could not render the product of that process obvious under 35 U.S.C. 103). The Federal Circuit has pointed out that under United States law, a description that does not render a claimed invention obvious cannot sufficiently describe the invention for the purposes of the written description requirement of 35 U.S.C. 112. *Eli Lilly*, 119 F.3d at 1567, 43 USPQ2d at 1405. The fact that a great deal more than just a process is necessary to render a product invention obvious means that a great deal more than just a process is necessary to provide written description for a product invention.

Compare Fonar Corp. v. *General Electric Co.*, 107 F.3d 1543, 1549, 41 USPQ2d 1801, 1805 (Fed. Cir. 1997) ("As a general rule, where software constitutes part of a best mode of carrying out an invention, description of such a best mode is satisfied by a disclosure of the functions of the software. This is because, normally, writing code for such software is within the skill of the art, not requiring undue experimentation, once its functions have been disclosed * * *. Thus, flow charts or source code listings are not a requirement for adequately disclosing the functions of software.").

14. *See, e.g., Fujikawa* v. *Wattanasin*, 93 F.3d 1559, 1571, 39 USPQ2d 1895, 1905 (Fed. Cir. 1996) (a "laundry list" disclosure of every possible moiety does not constitute a written description of every species in a genus because it would not "reasonably

lead" those skilled in the art to any particular species); *In re Ruschig*, 379 F.2d 990, 995, 154 USPQ 118, 122–23 (CCPA 1967) ("If n-propylamine had been used in making the compound instead of n-butylamine, the compound of claim 13 would have resulted. Appellants submit to us, as they did to the board, an imaginary specific example patterned on specific example 6 by which the above butyl compound is made so that we can see what a simple change would have resulted in a specific supporting disclosure being present in the present specification. The trouble is that there is no such disclosure, easy though it is to imagine it.").

15. 35 U.S.C. 132 and 251. *See also In re Rasmussen*, 650 F.2d 1212, 1214, 211 USPQ 323, 326 (CCPA 1981). *See* Manual of Patent Examining Procedure (MPEP) §§ 2163.06–2163.07 (7th Ed., July 1998) for a more detailed discussion of the written description requirement and its relationship to new matter.

16. The claims as filed in the original specification are part of the disclosure and therefore, if an application as originally filed contains a claim disclosing material not found in the remainder of the specification, the applicant may amend the specification to include the claimed subject matter. *In re Benno*, 768 F.2d 1340, 226 USPQ 683 (Fed. Cir. 1985).

17. *See, e.g., In re Lukach*, 442 F.2d 967, 169 USPQ 795 (CCPA 1971) (subgenus range was not supported by generic disclosure and specific example within the subgenus range); *In re Smith*, 458 F.2d 1389, 1395, 173 USPQ 679, 683 (CCPA 1972) (a subgenus is not necessarily described by a genus encompassing it and a species upon which it reads).

18. *In re Oda*, 443 F.2d 1200, 170 USPQ 260 (CCPA 1971). With respect to the correction of sequencing errors in applications disclosing nucleic acid and/or amino acid sequences, it is well know that sequencing errors are a common problem in molecular biology. *See, e.g.*, Richterich, Peter, "Estimation of Errors in 'Raw' DNA Sequences: A Validation Study," *Genome Research*, 8:251–259 (1998). If an application as filed includes sequence information and references a deposit of the sequenced material made in accordance with the requirements of 37 CFR 1.801 *et seq.*, corrections of minor errors in the sequence may be possible based on the argument that one of skill in the art would have resequenced the deposited material and would have immediately recognized the minor error. Deposits made after the filing date can only be relied upon to provide support for the correction of sequence information if applicant submits a statement in compliance with 37 CFR 1.804 stating that the biological material which is deposited is a biological material specifically defined in the application as filed.

19. *See, e.g., Gentry Gallery, Inc.* v. *Berkline Corp.*, 134 F.3d 1473, 45 USPQ2d 1498 (Fed. Cir. 1998) (claims to a section sofa comprising, *inter alia*, a console and a control means were held invalid for failing to satisfy the written description requirement where the claims were broadened by removing the location of the control means.); *Johnson Worldwide Associates Inc.* v. *Zebco Corp.*, 175 F.3d 985, 993, 50 USPQ2d 1607, 1613 (Fed. Cir. 1999) (In *Gentry Gallery*, the "court's determination that the patent disclosure did not support a broad meaning for the disputed claim terms was premised on clear statements in the written description that described the location of a claim element—the 'control means'—as 'the only possible location' and that variations were 'outside the stated purpose of the invention.' *Gentry Gallery*, 134 F.3d at 1479, 45 USPQ2d at 1503. *Gentry Gallery*, then, considers the situation where the patent's disclosure makes crystal clear that a particular (*i.e.*, narrow) understanding of a claim term is an 'essential element of [the inventor's] invention.' "); *Tronzo* v. *Biomet, Inc.*, 156 F.3d 1154, 1159, 47 USPQ2d 1829, 1833 (Fed. Cir. 1998) (claims to generic cup shape were not entitled to filing date of parent application which disclosed "conical cup" in view of the disclosure of the parent application stating the advantages and importance of the conical shape.).

20. *See Gentry Gallery*, 134 F.3d at 1480, 45 USPQ2d at 1503; *In re Sus*, 306 F.2d 494, 134 USPQ 301 (CCPA 1962) ("[O]ne skilled in this art would not be taught by the written description of the invention in the specification that any 'aryl or substituted aryl radical' would be suitable for the purposes of the invention but rather that only certain aryl radicals and certain specifically substituted aryl radicals [*i.e.*, aryl azides] would be suitable for such purposes."). A claim which omits matter disclosed to be essential to the invention as described in the specification or in other statements of record may also be subject to rejection under 35 U.S.C. § 112, ¶ 1 as not enabling, or under 35 U.S.C. 112, ¶ 2. *See In re Mayhew*, 527 F.2d 1229, 188 USPQ 356 (CCPA 1976); *In re Venezia*, 530 F.2d 956, 189 USPQ 149 (CCPA 1976); and *In re Collier*, 397 F.2d 1003, 158 USPQ 266 (CCPA 1968). *See also Reiffin* v. *Microsoft Corp.*, 48 USPQ2d 1274, 1277 (N.D. Cal. 1998) and MPEP § 2172.01.

21. *See, e.g., Vas-Cath, Inc.*, 935 F.2d at 1563–64, 19 USPQ2d at 1117.

22. *Wertheim*, 541 F.2d at 262, 191 USPQ at 96.

23. *See* MPEP §§ 714.02 and 2163.06 ("Applicant should * * * specifically point out the support for any amendments made to the disclosure."); and MPEP § 2163.04 ("If applicant amends the claims and points out where and/or how the originally filed disclosure supports the amendment(s), and the examiner finds that the disclosure does not reasonably convey that the inventor had possession of the subject matter of the amendment at the time of the filing of the application, the examiner has the initial burden of presenting evidence or reasoning to explain why persons skilled in the art would not recognize in the disclosure a description of the invention defined by the claims.").

24. *See In re Smith*, 458 F.2d 1389, 1395, 173 USPQ 679, 683 (CCPA 1972) ("Precisely how close [to the claimed invention] the description must come to comply with § 112 must be left to case-by-case development."); *In re Wertheim*, 541 F.2d at 262, 191 USPQ at 96 (inquiry is primarily factual and depends on the nature of the invention and the amount of knowledge imparted to those skilled in the art by the disclosure).

25. *See, e.g., In re Morris*, 127 F.3d 1048, 1053–54, 44 USPQ2d 1023, 1027 (Fed. Cir. 1997).

26. "Preamble language" is that language in a claim appearing before the transitional phase, e.g., before "comprising," "consisting essentially of," or "consisting of."

27. The transitional term "comprising" (and other comparable terms, *e.g.*, "containing," "including," and "having") is "open-ended—it covers the expressly recited subject matter, alone or in combination with unrecited subject matter. *See, e.g., Ex parte Davis*, 80 USPQ 448, 450 (Bd. App. 1948) ("comprising" leaves the "claim open for the inclusion of unspecified ingredients even in major amounts"), *quoted with approval in Moleculon Research Corp* v. *CBS, Inc.*, 793 F.2d 1261, 1271, 229 USPQ 805, 812 (Fed. Cir. 1986). "By using the term 'consisting essentially of,' the drafter signals that the invention necessarily includes the listed ingredients and is open to unlisted ingredients that do not materially affect the basic and novel properties of the invention. A 'consisting essentially of' claim occupies a middle ground between closed claims that are written in a 'consisting of' format and fully open claims that are drafted in a 'comprising' format." *PPG Industries* v. *Guardian Industries*, 156 F.3d 1351, 1354, 48 USPQ2d 1351, 1353–54 (Fed. Cir. 1998). For search and examination purposes, absent a clear indication in the specification of what the basic and novel characteristics actually are, 'consisting essentially of' will be construed as equivalent to "comprising." *See, e.g., PPG*, 156 F.3d at 1355, 48 USPQ at 1355 ("PPG could have defined the scope of the phrase 'consisting essentially of' for purposes of its patent by making clear in its specification what it regarded as constituting a material change in the basic and novel characteristics of the invention.").

28. *See Pac-Tec Inc.* v. *Amerace Corp.*, 903 F.2d 796, 801, 14 USPQ2d 1871, 1876 (Fed. Cir. 1990) (determining that preamble language that constitutes a structural limitation is actually part of the claimed invention).

29. An applicant shows possession of the claimed invention by describing the claimed invention with all of its essential novel elements. *Lockwood*, 107 F.3d at 1572, 41 USPQ2d at 1966.

30. *See, e.g., Bell Communications Research, Inc.* v. *Vitalink Communications Corp.*, 55 F.3d 615, 620, 34 USPQ2d 1816, 1820 (Fed. Cir. 1995) ("[A] claim preamble has the import that the claim as a whole suggests for it."); *Corning Glass Works* v. *Sumitomo Elec. U.S.A., Inc.*, 868 F.2d 1251, 1257, 9 USPQ2d 1962, 1966 (Fed. Cir. 1989) (The determination of whether preamble recitations are structural limitations can be resolved only on review of the entirety of the application "to gain an understanding of what the inventors actually invented and intended to encompass by the claim.").

31. *See, e.g., Wang Labs.* v. *Toshiba Corp.*, 993 F.2d 858, 865, 26 USPQ2d 1767, 1774 (Fed. Cir. 1993).

32. *See, e.g., Hybritech Inc.* v. *Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1379–80, 231 USPQ 81, 90 (Fed. Cir. 1986).

33. *Pfaff* v. *Wells Electronics, Inc.*, 119 S.Ct. at 311, 48 USPQ2d at 1646 ("The word 'invention' must refer to a concept that is complete, rather than merely one that is 'substantially complete.' It is true that reduction to practice ordinarily provides the best evidence that an invention is complete. But just because reduction to practice is sufficient evidence of completion, it does not follow that proof of reduction to practice is necessary in every case. Indeed, both the facts of the *Telephone Cases* and the facts of this case demonstrate that one can prove that an invention is complete and ready for patenting before it has actually been reduced to practice.").

34. *Cooper* v. *Goldfarb*, 154 F.3d 1321, 1327, 47 USPQ2d 1896, 1901 (Fed. Cir. 1998). *See also UMC Elecs. Co.* v. *United States*, 816 F.2d 647, 652, 2 USPQ2d 1465, 1468 (Fed. Cir. 1987) ("[T]here cannot be a reduction to practice of the invention * * * without a physical embodiment which includes all limitations of the claim."); *Estee Lauder Inc.* v. *L'Oreal S.A.*, 129 F.3d 588, 593, 44 USPQ2d 1610, 1614 (Fed. Cir. 1997) ("[A] reduction to practice does not occur until the inventor has determined that the invention will work for its intended purpose."); *Mahurkar* v. *C.R. Bard Inc.*, 79 F.3d 1572, 1578, 38 USPQ2d 1288, 1291 (Fed. Cir. 1996) (determining that the invention will work for its intended purpose may require testing depending on the character of the invention and the problem it solves).

35. 37 CFR §§ 1.804, 1.809. *See also* endnote 6.

36. *See, e.g., Vas-Cath*, 935 F.2d at 1565, 19 USPQ2d at 1118 ("drawings alone may provide a 'written description' of an invention as required by § 112"); *In re Wolfensperger*, 302 F.2d 950, 133 USPQ 537 (CCPA 1962) (the drawings of applicant's specification provided sufficient written descriptive support for the claim limitation at issue); *Autogiro Co. of America* v. *United States*, 384 F.2d 391, 398, 155 USPQ 697, 703 (Ct. Cl. 1967) ("[I]n those instances where a visual representation can flesh out words, drawings may be used in the same manner and with the same limitations as the specification.").

37. *See Hybritech* v. *Monoclonal Antibodies*, 802 F.2d at 1384, 231 USPQ at 94; *Fonar Corp.* v. *General Electric Co.*, 107 F.3d at 1549, 41 USPQ2d at 1805 (source code description not required).

38. This is especially true for the mechanical and electrical arts. *See, e.g., Pfaff* v. *Wells Electronics*, 119 S.Ct. at 312, 48 USPQ2d at 1647.

39. For example, the presence of a restriction enzyme map of a gene may be relevant to a statement that the gene has been isolated. One skilled in the art may be able to determine when the gene disclosed is the same as or different from a gene isolated by another by comparing the restriction enzyme map. In contrast, evidence that the gene could be digested with a nuclease would not normally represent a relevant characteristic since any gene would be digested with a nuclease. Similarly, isolation of an mRNA and its expression to produce the protein of interest is strong evidence of possession of an mRNA for the protein.

Examples of identifying characteristics include a sequence, structure, binding affinity, binding specificity, molecular weight, and length. Although structural formulas provide a convenient method of demonstrating possession of specific molecules, other identifying characteristics or combinations of characteristics may demonstrate the requisite possession. For example, unique cleavage by particular enzymes, isoelectric points of fragments, detailed restriction enzyme maps, a comparison of enzymatic activities, or antibody cross-reactivity may be sufficient to show possession of the claimed invention to one of skill in the art. *See Lockwood*, 107 F.3d at 1572, 41 USPQ2d at 1966 ("written description" requirement may be satisfied by using "such descriptive means as words, structures, figures, diagrams, formulas, etc., that fully set forth the claimed invention").

However, a definition by function alone "does not suffice" to sufficiently describe a coding sequence "because it is only an indication of what the gene does, rather than what it is." *Eli Lilly*, 119 F.3 at 1568, 43 USPQ2d at 1406. *See also Fiers*, 984 F.2d at 1169–71, 25 USPQ2d at 1605–06 (discussing *Amgen Inc.* v. *Chugai Pharmaceutical Co.*, 927 F.2d 1200, 18 USPQ2d 1016 (Fed. Cir. 1991)).

40. If a claim limitation invokes 35 U.S.C. § 112, ¶ 6, it must be interpreted to cover the corresponding structure, materials, or acts in the specification and "equivalents thereof." See 35 U.S.C. 112, ¶ 6. *See also B. Braun Medical, Inc.* v. *Abbott Lab.*, 124 F.3d 1419, 1424, 43 USPQ2d 1896, 1899 (Fed. Cir. 1997). If the written description fails to set forth the supporting structure, material or acts corresponding to the means-(or step-) plus-function, the claim may not meet the requirement of 35 U.S.C. 112, ¶ 1. A means-(or step-) plus-function claim limitation satisfies 35 U.S.C. 112, ¶ 1 if: (1) The written description links or associates particular structure, materials, or acts to the function recited in a means-(or step-) plus-function claim limitation; or (2) it is clear based on the facts of the application that one skilled in the art would have known what structure, materials, or acts perform the function recited in a means-(or step-) plus-function limitation. In considering whether there is 35 U.S.C. § 112, ¶ 1 support for the claim limitation, the examiner must consider not only the original disclosure contained in the summary and detailed description of the invention portions of the specification, but also the original claims, abstract, and drawings. See the Interim Supplemental Examination Guidelines for Determining the Applicability of 35 U.S.C. 112 ¶ 6, 64 FR 41392 (July 30, 1999).

41. *See Hybritech Inc.* v. *Monoclonal Antibodies, Inc.*, 802 F.2d at 1384, 231 USPQ at 94.

42. *See, e.g., Vas-Cath*, 935 F.2d at 1563, 19 USPQ2d at 1116; *Martin* v. *Johnson*, 454 F.2d 746, 751, 172 USPQ 391, 395 (CCPA 1972) (stating "the description need not be in *ipsis verbis* [i.e., "in the same words"] to be sufficient").

43. A claim which is limited to a single disclosed embodiment or species is analyzed as a claim drawn to a single embodiment or species, whereas a claim which encompasses two or more embodiments or species within the scope of the claim is analyzed as a claim drawn to a genus. *See also* MPEP § 806.04(e).

44. 35 U.S.C. 112, ¶ 1. *Cf. Fields* v. *Conover*, 443 F.2d 1386, 1392, 170 USPQ 276, 280 (CCPA 1971) (finding a lack of written description because the specification lacked the "full, clear, concise, and exact written description" which is necessary to support the claimed invention).

45. For example, if the art has established a strong correlation between structure and function, one skilled in the art would be able to predict with a reasonable degree of confidence the structure of the claimed invention from a recitation of its function. Thus, the written description requirement may be satisfied through disclosure of function and minimal structure when there is a well-established correlation between structure and function. In contrast, without such a correlation, the capability to recognize or understand the structure from the mere recitation of function and minimal structure is highly unlikely. In this latter case, disclosure of function alone is little more than a wish for possession: it does not satisfy the written description requirement. *See Eli Lilly*, 119 F.3d at 1568, 43 USPQ2d at 1406 (written description requirement not satisfied by merely providing "a result that one might achieve if one made that invention"); *In re Wilder*, 736 F.2d 1516, 1521, 222 USPQ 369, 372–73 (Fed. Cir. 1984) (affirming a rejection for lack of written description because the specification does "little more than outline goals appellants hope the claimed invention achieves and the problems the invention will hopefully ameliorate"). *Compare Fonar*, 107 F.3d at 1549, 41 USPQ2d at 1805 (disclosure of software function adequate in that art).

46. *See, e.g., In re Hayes Microcomputer Products Inc. Patent Litigation*, 982 F.2d 1527, 1534–35, 25 USPQ2d 1241, 1246 (Fed. Cir. 1992) ("One skilled in the art would know how to program a microprocessor to perform the necessary steps described in the specification. Thus, an inventor is not required to describe every detail of his invention. An applicant's disclosure obligation varies according to the art to which the invention pertains. Disclosing a microprocessor capable of performing certain functions is sufficient to satisfy the requirement of section 112, first paragraph, when one skilled in the relevant art would understand what is intended and know how to carry it out.")

47. *See, e.g., Fiers* v. *Revel*, 984 F.2d at 1169, 25 USPQ2d at 1605; *Amgen Inc.* v. *Chugai Pharmaceutical Co.*, 927 F.2d 1200, 1206, 18 USPQ2d 1016, 1021 (Fed. Cir. 1991). Where the process has actually been used to produce the product, the written description requirement for a product-by-process claim is clearly satisfied; however, the requirement may not be satisfied where it is not clear that the acts set forth in the specification can be performed, or that the product is produced by that process.

48. *See, e.g., Amgen Inc.* v. *Chugai Pharmaceutical Co.*, 927 F.2d 1200, 1206, 18

USPQ2d 1016, 1021 (Fed. Cir. 1991)("A gene is a chemical compound, albeit a complex one, and it is well established in our law that conception of a chemical compound requires that the inventor be able to define it so as to distinguish it from other materials, and to describe how to obtain it. Conception does not occur unless one has a mental picture of the structure of the chemical, or is able to define it by its method of preparation, its physical or chemical properties, or whatever characteristics sufficiently distinguish it. It is not sufficient to define it solely by its principal biological property, *e.g.*, encoding human erythropoietin, because an alleged conception having no more specificity than that is simply a wish to know the identity of any material with that biological property. We hold that when an inventor is unable to envision the detailed constitution of a gene so as to distinguish it from other materials, as well as a method for obtaining it, conception has not been achieved until reduction to practice has occurred, *i.e.*, until after the gene has been isolated.")(citations omitted). In such instances the alleged conception fails not merely because the field is unpredictable or because of the general uncertainty surrounding experimental sciences, but because the conception is incomplete due to factual uncertainty that undermines the specificity of the inventor's idea of the invention. *Burroughs Wellcome Co.* v. *Barr Laboratories Inc.*, 40 F.3d 1223, 1229, 32 USPQ2d 1915, 1920 (Fed. Cir. 1994). Reduction to practice in effect provides the only evidence to corroborate conception (and therefore possession) of the invention. *Id.*

49. *See Eli Lilly*, 119 F.3d at 1568, 43 USPQ2d at 1406.

50. *See, e.g., Eli Lilly.*

51. For example, in the genetics arts, it is unnecessary for an applicant to provide enough different species that the disclosure will permit one of skill to determine the nucleic acid or amino acid sequence of another species from the application alone. The stochastic nature of gene evolution would make such a predictability nearly impossible. Thus, the Federal Circuit could not have intended that representative number requires predictability of sequences.

52. *See Wertheim*, 541 F.2d at 263, 191 USPQ at 97 ("[T]he PTO has the initial burden of presenting evidence or reasons why persons skilled in the art would not recognize in the disclosure a description of the invention defined by the claims."). *See also* MPEP § 2163.05.

53. *See* MPEP §§ 714.02 and 2163.06 ("Applicant should * * * specifically point out the support for any amendments made to the disclosure.").

54. *See, e.g., In re Wright*, 866 F.2d 422, 425, 9 USPQ2d 1649, 1651 (Fed. Cir. 1989)(Original specification for method of forming images using photosensitive microcapsules which describes removal of microcapsules from surface and warns that capsules not be disturbed prior to formation of image, unequivocally teaches absence of permanently fixed microcapsules and supports amended language of claims requiring that microcapsules be "not permanently fixed" to underlying surface, and therefore meets description requirement of 35 U.S.C. 112.).

55. *See, e.g., In re Robins*, 429 F.2d 452, 456–57, 166 USPQ 552, 555 (CCPA 1970) ("[W]here no explicit description of a generic invention is to be found in the specification * * * mention of representative compounds may provide an implicit description upon which to base generic claim language."); *In re Smith*, 458 F.2d 1389, 1395, 173 USPQ 679, 683 (CCPA 1972)(a subgenus is not necessarily implicitly described by a genus encompassing it and a species upon which it reads).

56. *See, e.g., In re Robertson*, 169 F.3d 743, 745, 49 USPQ2d 1949, 1950–51 (Fed. Cir. 1999)("To establish inherency, the extrinsic evidence 'must make clear that the missing descriptive matter is necessarily present in the thing described in the reference, and that it would be so recognized by persons of ordinary skill. Inherency, however, may not be established by probabilities or possibilities. The mere fact that a certain thing may result from a given set of circumstances is not sufficient.'") (citations omitted).

57. When an explicit limitation in a claim "is not present in the written description whose benefit is sought it must be shown that a person of ordinary skill would have understood, at the time the patent application was filed, that the description requires that limitation." *Hyatt* v. *Boone*, 146 F.3d 1348, 1353, 47 USPQ2d 1128, 1131 (Fed. Cir. 1998).

58. *See, e.g., Johnson Worldwide Associates Inc.* v. *Zebco Corp.*, 175 F.3d at 993, 50 USPQ2d at 1613; *Gentry Gallery, Inc.* v. *Berkline Corp.*, 134 F.3d at 1479, 45 USPQ2d at 1503; *Tronzo* v. *Biomet, Inc.*, 156 F.3d at 1159, 47 USPQ2d at 1833; and *Reiffin* v. *Microsoft Corp.*, 48 USPQ2d at 1277.

59. *See, e.g., In re Marzocchi*, 439 F.2d 220, 224, 169 USPQ 367, 370 (CCPA 1971).

60. *Wertheim*, 541 F.2d at 262, 191 USPQ at 96.

61. *See In re Rasmussen*, 650 F.2d at 1214, 211 USPQ at 326.

62. *See In re Alton*, 76 F.3d 1168, 1176, 37 USPQ2d 1578, 1584 (Fed. Cir. 1996).

Dated: December 16, 1999.

Q. Todd Dickinson,

Assistant Secretary of Commerce and Commissioner of Patents and Trademarks.

[FR Doc. 99–33053 Filed 12–20–99; 8:45 am]

BILLING CODE 3510–16–P

DEPARTMENT OF COMMERCE

Patent and Trademark Office

[Docket No. 991027289–9289–01]

RIN 0651–AB09

Revised Utility Examination Guidelines; Request for Comments

AGENCY: Patent and Trademark Office, Commerce.

ACTION: Notice and request for public comments.

SUMMARY: The Patent and Trademark Office (PTO) requests comments from any interested member of the public on the following Revised Utility Examination Guidelines. The PTO is publishing a revised version of guidelines to be used by Office personnel in their review of patent applications for compliance with the utility requirement based on comments received in response to the Request for Comments on Interim Guidelines for Examination of Patent Applications Under the 35 U.S.C. 112, ¶ 1 "Written Description" Requirement; Extension of Comment Period and Notice of Hearing, 63 FR 50887 (September 23, 1998). These Revised Utility Guidelines will be used by PTO personnel in their review of patent applications for compliance with the "utility" requirement of 35 U.S.C. 101. This revision supersedes the Utility Examination Guidelines that were published at 60 FR 36263 (1995) and at 1177 O.G. 146 (1995).

DATES: Written comments on the Revised Utility Examination Guidelines will be accepted by the PTO until March 22, 2000.

ADDRESSES: Written comments should be addressed to Box 8, Commissioner of Patents and Trademarks, Washington, DC 20231, marked to the attention of Mark Nagumo, or to Box Comments, Assistant Commissioner for Patents, Washington, DC 20231, marked to the attention of Linda S. Therkorn. Alternatively, comments may be submitted to Mark Nagumo via facsimile at (703)-305–9373 or by electronic mail addressed to "mark.nagumo@uspto.gov"; or to Linda Therkorn via facsimile at (703) 305–8825 or by electronic mail addressed to "linda.therkorn@uspto.gov."

FOR FURTHER INFORMATION CONTACT: Mark Nagumo by telephone at (703) 305–8666, by facsimile at (703) 305–9373, by electronic mail "mark.nagumo@uspto.gov," or by mail marked to his attention addressed to the Commissioner of Patents and Trademarks, Box 8, Washington, DC 20231; or Linda Therkorn by telephone at (703) 305–9323, by facsimile at (703) 305–8825, by electronic mail at "linda.therkorn@uspto.gov," or by mail marked to her attention addressed to Box Comments, Assistant Commissioner of Patents and Trademarks, Washington, DC 20231.

SUPPLEMENTARY INFORMATION: The PTO requests comments from any interested member of the public on the following Revised Utility Examination Guidelines. As of the publication date of this notice, this revision will be used by PTO personnel in their review of patent

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Two Tumor Necrosis Factor-binding Proteins Purified from Human Urine

EVIDENCE FOR IMMUNOLOGICAL CROSS-REACTIVITY WITH CELL SURFACE TUMOR NECROSIS FACTOR RECEPTORS*

(Received for publication, August 4, 1989)

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From the Department of Molecular Genetics and Virology, The Weizmann Institute of Science, Rehovot 76100, Israel

Two proteins which specifically bind tumor necrosis factor (TNF) were isolated from human urine by ligand (TNF)-affinity purification, followed by reversed phase high performance liquid chromatography. The molecular weights of the two proteins, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, were similar (about 30,000). Both proteins provided protection against the cytocidal effect of TNF *in vitro* and both bound TNF-α more effectively than TNF-β. Antibodies raised against each of the proteins had an inhibitory effect on the binding of TNF to cells, suggesting that both proteins are structurally related to the TNF receptors. However, the two proteins differed in NH_2-terminal amino acid sequences: Asp-Ser-Val-Cys-Pro- in one and Val-Ala-Phe-Thr-Pro- in the other. The NH_2-terminal sequence of the former protein was invariable, while that of the latter was truncated to varying degrees. The two proteins were also immunologically distinct. The relative efficacy of antisera against the two proteins in inhibiting the binding of TNF to cells varied markedly from one line of cells to another. Evidence has been presented recently for the existence of two distinct molecular species of cell surface receptors for TNF and for differential expression of these two receptors by cells of different lines. The findings presented in this study are consistent with the notion that the urinary TNF-binding proteins constitute soluble forms of the two molecular species of the cell surface TNF receptors.

Effects of tumor necrosis factor (TNF)[1] on cell function contribute, in a number of ways, to the defense of the organism against infectious agents and to recovery from injury. However, in certain pathological situations, some of these same effects may become detrimental, causing even more harm than the pathogen which induced the formation of TNF (1, 2). There is therefore particular interest in elucidating the mechanisms whereby these effects are regulated.

In the sequence of molecular events that take place in the response of the cell to TNF, the most readily accessible to modulation is the initiation of this process triggered by the binding of TNF to its cell surface receptors. One way of modulating this interaction is by inducing changes in the expression of the TNF receptors. Both the number and affinity of the receptors were found to vary in response to certain regulators (3–7). Furthermore, there may be also variation in the kind of receptor molecules which cells can express. Recent studies suggest that there are two different molecular species of TNF receptors and that these are expressed differentially by cells of different lines.[2]

A different way of modulating the binding of TNF to its receptors was reported in several recent studies, in which a protective effect of proteins found in human urine against the cytocidal activity of TNF was described (8–10). This protection could be related to the function of certain minor constitutory proteins of the urine which bind TNF and thus decrease its availability to the TNF receptors. One of these proteins has been purified (10–12). Here, we present evidence that there are two different TNF-binding proteins in the urine. Evidence is also presented suggesting that these proteins may be structurally related to the two cell surface TNF receptors. These findings raise the possibility that cells can produce receptors for TNF also in a soluble form and that, when presented in such form to the cell, the receptors can compete for TNF with the membrane-associated receptors and thus function as inhibitors for TNF activity.

MATERIALS AND METHODS

Cells

Cells of the HeLa (13), MCF7 (14), K562 (15), and U937 (16) lines were cultured in RPMI 1640 medium supplemented with 100 μg/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated fetal calf serum.

Cytokines

The recombinant human TNF-α (rhuTNF-α, 3.2×10^7 units/mg protein) used for the affinity purification of the TNF-binding proteins was purchased from Pharma Biotechnologie, Hanover, West Germany. The rhuTNF-α used for all other purposes (6×10^7 units/mg protein) and the recombinant human TNF-β (rhuTNF-β, lymphotoxin, 1.2×10^8 units/mg protein) were kindly provided by Dr. G. Adolf of the Boehringer Institute, Vienna, Austria. Recombinant human IL-1α (rIL-1α, 2.5×10^8 units/mg protein), consisting of the

* This work was supported in part by grants from the Israel Ministry of Science, The National Council for Research and Development, Israel, and from Inter-Lab, Ltd., Nes-Ziona, Israel. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence and reprint requests should be addressed.

[1] The abbreviations used are: TNF, tumor necrosis factor; ELISA, enzyme-linked immunosorbent assay; IFN, interferon; HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; TBP, TNF-binding protein; SDS, sodium dodecyl sulfate; IL-1, interleukin-1; IL-6, interleukin-6; PBS, phosphate-buffered saline; r, recombinant; hu, human; BSA, bovine serum albumin.

[2] H-P. Hohmann, R. Remmy, M. Brockhaus, and A. P. G. M. Van Loon, an abstract presented in the 2nd International Conference on TNF and Related Cytokines, Napa, CA, January 15–20, 1989; and M. Brockhaus, H. Loetscher, H-P. Hohmann, and W. Huziker, *ibid.*

154 carboxyl-terminal amino acids of the 271-amino acid human IL-1 precursor, was a gift of Drs. A. Stern and P. T. Lomedico (Hoffman-La Roche, Nutley, NJ). Recombinant human interferon-γ (rIFN-γ, 5×10^7 units/mg protein) and recombinant human interleukin 6 (rIL-6, 3×10^6 units/mg protein, as determined by the hybridoma growth factor assay) were a gift from InterPharm Laboratories, Ness Ziona, Israel. Radiolabeling of TNF-α (to 140 μCi/μg), TBPI (to 170 μCi/μg), and TBPII (to 290 μCi/μg) was done by the chloramine-T method, as described previously (4).

Antibodies

Three-month-old rabbits (New Zealand White) were immunized with TBPI and TBPII, at doses of 5 and 20 μg, respectively, according to the following protocol: The proteins were first injected subcutaneously as emulsion in complete Freund's adjuvant. After 3 weeks, the animals were injected intramuscularly with an emulsion of the proteins in incomplete Freund's adjuvant and then twice, subcutaneously, at 1-week intervals with the proteins in PBS solution.

Assays for the TNF-binding Proteins

Bioassays—Quantitation of the protective effect of the TNF-binding proteins against TNF cytotoxicity and of their inhibitory effect on the binding of radiolabeled TNF to cells was performed as described before (10). When using suspended cells (K562 and U937), washing of unbound radiolabeled TNF was achieved by repeated centrifugation (6). All assays were performed in duplicates.

Binding of TBP to Cytokines—The solid phase assay for the binding of various cytokines to the purified TNF-binding protein was performed as described before (10).

Assays for Antibodies against TNF-binding Proteins

Western Blotting Analysis—Proteins to be tested were applied to SDS-PAGE (12% acrylamide gels, 1 mm thickness) and then blotted electrophoretically onto a nitrocellulose sheet (Schleicher & Schull, F.R.G), using the Bio-Rad mini-protean II dual slab cell and mini-trans-blot devices. The nitrocellulose sheet was incubated for 2 h with 10% milk (v/v) in PBS containing 0.1% sodium azide and 0.05% Tween 20 and was then briefly rinsed in PBS containing 0.05% Tween 20 (PBS-Tween). It was then incubated for 2 h with the test antibodies in a multiflane device followed by incubation for 1 h with ^{125}I-labeled protein A (Amersham Corp., 40 μCi/μg, 5×10^5 cpm/ml). After washing off the unbound material, the nitrocellulose sheet was exposed to autoradiography. All incubations were carried out at room temperature. All reagents were applied in 10% milk in PBS containing sodium azide and Tween 20, as above.

ELISA—Ninety-six-well ELISA plates (Nunc, Denmark) were coated with 1 μg/ml of TBPI, TBPII, or BSA as a control, in PBS containing 0.02% sodium azide, by incubating the plates for 2 h at 37 °C followed by overnight incubation at 4 °C. The plates were then rinsed with PBS-Tween and incubated for 2 h with a solution of 0.5% BSA in PBS-Tween. After rinsing again with PBS-Tween the wells were incubated for 2 h with the test antibodies, then rinsed again and incubated for 2 h with purified goat antibody to rabbit IgG, conjugated to horseradish peroxidase (Biomakor, Israel). After a further washing step with PBS-Tween, the activity of horseradish peroxidase-conjugated antibody which bound to the plate was determined using 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) as a substrate. The enzymatic product was determined colorimetrically at 600 nm. All incubations were performed at 37 °C.

Effect of the Antibodies on Binding of TNF to Cells—The antisera to TBPI and TBPII were diluted in PBS containing 0.5% BSA and 0.1% sodium azide and then added either directly or after incubation for 30 min at 4 °C with a sample of TBP (Fig. 7) for 2 h to the test cells. The cells were then rinsed and tested for binding of TNF as described elsewhere (6).

Purification of the TNF-binding Proteins

Ligand (TNF)-affinity Purification—Proteins of pooled urine of healthy postmenopausal women were concentrated 600-fold as described before (10). A sample of 250 ml of the concentrate was applied at a flow rate of 0.25 ml/min to a column constructed of 0.5 ml of Affi-Gel 10 (Bio-Rad) to which 3.5 mg of rhuTNF-α (Pharma Biotechnologie, Hannover) was coupled. The column was then washed with PBS until all unbound proteins were removed, and the bound proteins were eluted by applying a solution containing 25 mM citric acid, 100 mM NaCl, and 0.02% sodium azide, at pH 2.5.

Reverse Phase HPLC—The proteins eluted from the TNF affinity column were applied to an Aquapore RP300 column (4.6 × 30 mm, Brownlee Labs) which was preequilibrated with 0.3% aqueous trifluoroacetic acid. The column was washed with the above solution at a flow rate of 0.5 ml/min until all unbound proteins were removed, and then eluted with a gradient of acetonitrile concentrations in 0.3% aqueous trifluoroacetic acid, as described before (10). Fractions of 0.5 ml were collected.

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RESULTS

In a prior study we described the isolation of a protein from human urine that could protect cultured cells from the cytocidal effect of TNF. The protein was purified to homogeneity in a series of chromatographic steps. It was found to bind TNF (10). Since the recovery of bioactivity in that procedure was low, the extent to which this protein contributes to the total protective activity of the unfractionated preparation of urinary proteins could not be determined. Therefore we attempted to isolate the protein in a more direct way, by affinity purification on a column of immobilized TNF.

Application of crude urinary proteins on such a column resulted in complete depletion of their protective activity, suggesting that this activity is fully mediated by TNF-binding protein(s) (Fig. 1 and Table I). The proteins which bound to the column could be eluted by decreasing the pH. The specific activity of the eluted proteins was about 20,000-fold higher than that of the crude urinary proteins. In SDS-PAGE analysis most of the proteins in the eluate migrated as a single broad band with apparent molecular size of 30,000 ± 2,000 (Fig. 2*A*). The affinity-purified proteins were further fractionated by reversed phase HPLC. The proteins were applied to an Aquapore RP300 column and eluted with a gradient of acetonitrile concentrations in the presence of 0.3% trifluoroacetic acid. They resolved into two active components; one (TBPI), eluting at 27% acetonitrile, similarly to what was reported previously (10, 11). The second protein (TBPII) eluted at a somewhat higher concentration of acetonitrile (31%) (Fig. 3).

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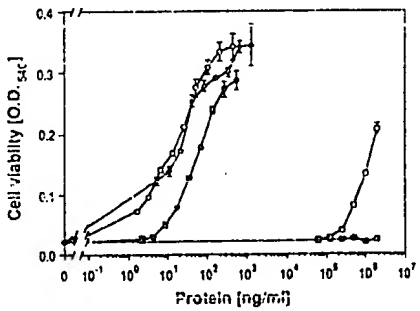

FIG. 1. **Titration of the bioactivity of the TNF-binding proteins at differing stages of purification.** □, unfractionated urinary proteins; ▨, effluent of the ligand (TNF)-affinity column; ◇, eluate of the ligand (TNF)-affinity column; ○, pooled fractions of TBPI following HPLC (fractions 19–21 in the experiment depicted in Fig. 3); ●, pooled fractions of TBPII following HPLC (fractions 24–27, Fig. 3). Purification of the TNF-binding proteins and quantitation of their protective effect against the cytotoxicity of TNF were carried out as described under "Materials and Methods."

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Application of crude urinary proteins on such a column resulted in complete depletion of their protective activity, suggesting that this activity is fully mediated by TNF-binding protein(s) (Fig. 1 and Table I). The proteins which bound to the column could be eluted by decreasing the pH. The specific activity of the eluted proteins was about 20,000-fold higher than that of the crude urinary proteins. In SDS-PAGE analysis most of the proteins in the eluate migrated as a single broad band with apparent molecular size of 30,000 ± 2,000 (Fig. 2*A*). The affinity-purified proteins were further fractionated by reversed phase HPLC. The proteins were applied to an Aquapore RP300 column and eluted with a gradient of acetonitrile concentrations in the presence of 0.3% trifluoroacetic acid. They resolved into two active components; one (TBPI), eluting at 27% acetonitrile, similarly to what was reported previously (10, 11). The second protein (TBPII) eluted at a somewhat higher concentration of acetonitrile (31%) (Fig. 3).

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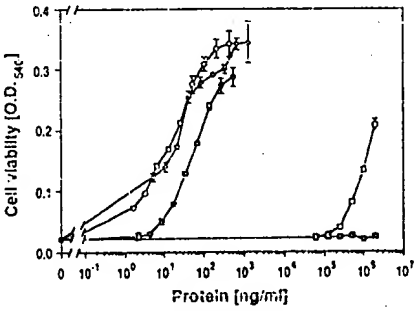

FIG. 1. **Titration of the bioactivity of the TNF-binding proteins at differing stages of purification.** □, unfractionated urinary proteins; ▨, effluent of the ligand (TNF)-affinity column; ◇, eluate of the ligand (TNF)-affinity column; ○, pooled fractions of TBPI following HPLC (fractions 19–21 in the experiment depicted in Fig. 3); ●, pooled fractions of TBPII following HPLC (fractions 24–27, Fig. 3). Purification of the TNF-binding proteins and quantitation of their protective effect against the cytotoxicity of TNF were carried out as described under "Materials and Methods."

TABLE I
Purification of the two TNF-binding proteins

Purification step	Protein	Protective activity		Specific activity	Purification
	mg	*units*[a]	%	*units*[a]*/mg*	*-fold*
Crude urinary proteins	9,400	117,200	100	12.4	
Affinity purification					
Flow-through	9,300	Not detectable			
Eluted proteins	0.36	98,600	84.2	273,800	22,000
Reversed phase HPLC					
Total	0.217	31,200	26.6	143,400	11,600
TBPI	0.070	22,300	19.0	318,600	
TBPII	0.147	8,900	7.6	60,700	

[a] A unit of protective activity was defined as the amount of TNF-binding proteins in whose presence the number of cells remaining viable, under the conditions of the assay for the protective effect of the proteins against TNF cytotoxicity, was doubled (10).

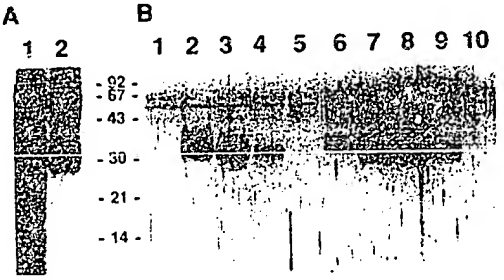

FIG. 2. **SDS-PAGE analysis of the crude and purified preparations of the TNF-binding proteins.** *A*, affinity purification step: *1*, unfractionated urinary proteins; *2*, proteins eluted from the TNF column by the low pH buffer. *B*, purification by reverse phase HPLC: *1–4*, TBPI (fractions 18–21 in the experiment of Fig. 3); *5–9*, TBPII (fractions 23–27); *10*, sample buffer. Migration of molecular weight markers (Pharmacia) is also depicted. Analysis was made under reducing conditions. The two bands migrating somewhat further than the 67 kDa molecular mass marker, which are particularly prominent in *lane 2*, are also detectable in *lane 10*, where only sample buffer was applied. They reflect the presence of some contaminants in the β-mercaptoethanol.

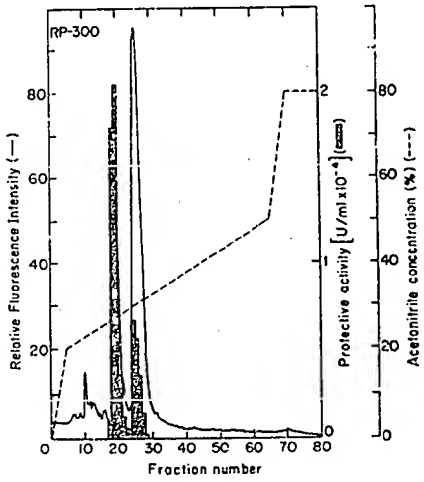

FIG. 3. **Reversed phase HPLC of the TBPs.** Ligand (TNF)-affinity column eluate was applied to an Aquapore RP300 column. Elution was performed with a gradient of acetonitrile in 0.3% aqueous trifluoroacetic acid (---) as described before (10). Fractions were examined for bioactivity (▨) and protein (——) content.

bind TNF-α specifically. Excess TNF-α or TNF-β, although less effectively, competed for this binding. Several other cytokines (IL-1, IL-6, IFN-γ, Table II) did not compete. In SDS-PAGE analysis both TBPI and TBPII exhibited heterogeneity in molecular size, comparable to that observed for the protein applied on the reverse phase column. Some variation in molecular size, from one eluted fraction to another, could be observed (Fig. 2*B*).

In spite of this variation in molecular size, NH_2-terminal microsequence analysis of TBPI revealed just a single NH_2-terminal sequence: Asp-Ser-Val-Cys-Pro-, identical to that reported for this protein before (10, 11). On the other hand, in TBPII, a number of NH_2-terminal sequences, all different from that of TBPI, could be observed. Furthermore, the quantitative proportions of these different sequences varied from fraction to fraction. It was found that this variation in sequence was not due to coexistence of different proteins in the TBPII preparation, but rather reflected truncation in the same protein to varying degrees at its NH_2 terminus. Thus in the experiment shown in Fig. 2, NH_2-terminal sequence analysis of the protein of fraction 27 revealed the sequence: Val-Ala-Phe-Thr-Pro-, but also, and in even higher amounts, a sequence shorter by three amino acids: Thr-Pro- and, in still higher amounts, a sequence which lacked just two of the terminal amino acids: Phe-Thr-Pro-. On the other hand, in fraction 28, the sequence Val-Ala-Phe-Thr-Pro- was the major one; but, also in lower amounts the sequence Phe-Thr-Pro- was detected; and, in even lower amounts, the sequence Thr-Pro-.

TABLE II
Binding of the two TNF-binding proteins to TNF-α and the effect of competitive cytokines

Proteins applied for competition for TBP binding[a]	Amounts of bound protein	
	^{125}I-TBPI	^{125}I-TBPII
	cpm	
	27,700 (± 2,000)	17,334 (± 1,230)
rhuTNF-α	1,050 (± 140)	2,400 (± 174)
rhuTNF-β	21,000 (± 850)	6,240 (± 230)
rIL-1α	28,100 (± 460)	17,840 (± 890)
rIL-6	27,050 (± 570)	18,570 (± 1,120)
rIFN-γ	28,050 (± 1,050)	18,470 (± 1,430)
TBPI	1,900 (± 170)	2,240 (± 160)
TBPII	ND[b]	2,005 (± 150)

[a] All proteins were applied at a concentration of 10 μg/ml.
[b] ND = not determined.

To explore further the interrelation between TBPI and TBPII, we examined the extent of their immunological cross-reactivity. As shown in Fig. 4, in Western blotting analysis,

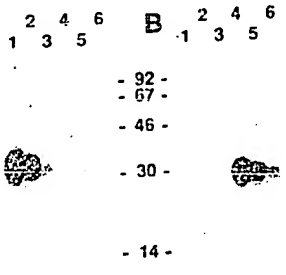

FIG. 4. **Binding of TBPI and TBPII antisera to the TNF-binding proteins as analyzed by Western blotting.** TBPI (*A, lanes 1–6*) and TBPII (*B, lanes 1–6*) were applied to SDS-PAGE at 2 μg/lane, together with 2 μg of BSA. Following electrophoresis, the proteins were blotted electrophoretically to a nitrocellulose sheet which was then incubated with antiserum to TBPI (*lanes 1–3*) or to TBPII (*lanes 4–6*) at the following dilutions: *lanes 1* and *4*, 1:100; *lanes 2* and *5*, 1:500; *lanes 2* and 6, 1:2500. After incubation with the antibodies the nitrocellulose sheet was incubated with ^{125}I-labeled protein A as described under "Materials and Methods."

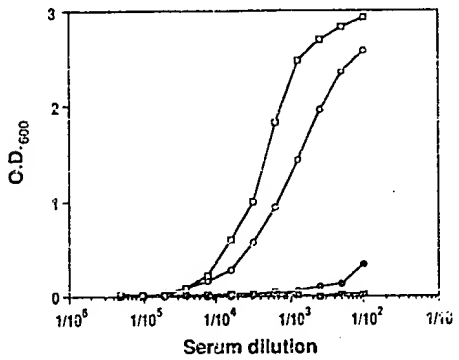

FIG. 5. **ELISA, for the binding of antisera against TBPI and TBPII to the two species of TBP.** The binding of □, antiserum against TBPI to TBPI; ▩, antiserum against TBPI to TBPII; ○, antiserum against TBPII to TBPII; and ●, antiserum against TBPII to TBPI is presented in terms of the absorbance of the color product in the horseradish peroxidase assay. Control values (binding of the antisera to BSA) were subtracted from all readings.

antisera raised in rabbits against the two proteins recognized just that species of TBP against which they had been raised. Similarly, using ELISA, the antiserum against TBPI was found to react with TBPI at a dilution of up to 1:25,000, but did not react with TBPII even at a dilution of 1:100. Conversely, the antiserum raised against TBPII bound TBPII at up to a dilution of 1:25,000 (Fig. 5). In this sensitive and quantitative assay, some binding of the antiserum against TBPII to TBPI could be observed, but only to a small extent and at a low dilution of the serum (1:400). This binding was fully abolished when the antiserum was incubated, before immunoassay, with TBPI, but it was not affected by preincubation of the serum with TBPII (not shown). Our data, therefore, do not appear to reflect immunological cross-reactivity between TBPI and TBPII, but rather the presence of low amounts of antibodies to TBPI in the antiserum preparation, apparently due to the presence of some contaminating TBPI in the TBPII sample used for immunization. Such contamination is not unexpected, since in the final purification step of the TNF binding proteins the two proteins elute from the reversed phase HPLC column adjacent to each other (see below).

To examine the relation of the two urinary TNF-binding proteins to cell surface receptors for TNF, which according to recent evidence also exist as two distinct molecular species,[2] we tested the effect of the antisera to the two proteins on the binding of TNF to its receptors. Both antisera had a marked inhibitory effect (Fig. 6). Since the effect was observed in the cold (4 °C) and in the presence of sodium azide, which was applied to suppress metabolic activities in the cell, it clearly was not due to any induced change in expression of the TNF receptors. Rather it appears to reflect direct interaction of the antibodies with the receptors resulting in interference with the binding of TNF to them. The relative effectivity of the antibodies against the two TBPs varied widely, depending on the cell type. In cells of the histiocytic lymphoma line, U937, the antiserum against TBPII had a marked inhibitory effect on TNF binding, while the antiserum to TBPI had no effect. In the chronic myeloid leukemia K562 cells, both antisera inhibited the binding of TNF, although to a different extent. Antibodies to TBPII abolished the binding completely while the antibodies to TBPI decreased TNF binding by only 20%, even when applied at high concentrations. In the HeLa, cervical carcinoma, and MCF 7 breast carcinoma cells, TNF binding was blocked effectively by the antiserum to TBPI; the antiserum to TBPII was also inhibitory, but only at much higher concentrations.

Since our quantitative study of the binding activities of the two antisera, using ELISA, revealed that the antiserum to TBPII contains also small amounts of antibodies to TBPI (Fig. 5), we preincubated samples of the antisera with either TBPI or TBPII, to determine whether, and to what extent, such contamination could account for the effects of the two antisera on TNF binding (Fig. 7). We found that 1) the inhibitory effect of the antiserum to TBPII on the binding of TNF to HeLa and MCF 7 cells could indeed be related to the effect of contaminating antibodies to TBPI in this antiserum. The inhibition was fully abolished when the antiserum against TBPII was preincubated with TBPI, but it was not affected by preincubation of the antiserum with TBPII at a concentration which abolished the effect of this antiserum on U937 cells. 2) The partial inhibition of TNF binding to the K562 cells by the antiserum to TBPI was due to a genuine effect of antibodies against this protein. It was not affected by preincubation of the antiserum with TBPII and was fully blocked by preincubation with TBPI. It thus appears that the K562 cells express both those receptor molecules which are recognized by antibodies to TBPII and, to a lesser extent, also receptors which are recognized by antibodies to TBPI.

These findings suggest that TBPI and TBPII display immunological cross-reactivity with the cell surface receptors for TNF. The findings are consistent with another recent study which suggests that the U937 and K562 cells express TNF receptors which are different from those expressed by the HeLa and MCF 7 cells[2] and indicate that there are cells (like K562) on which both kinds of receptors are expressed.

DISCUSSION

One of the two proteins whose purification is described in this study (TBPI) is identical to a TNF-binding protein we isolated previously from human urine by a chromatographic procedure (10). Purification of this protein has been described recently also by Olsson *et al.* (11), and it may also be identical to a "TNF inhibitor" purified from urine by Seckinger *et al.* (12). When isolated by a multistep chromatographic procedure, the TNF-binding protein turned out to be somewhat smaller than after ligand affinity purification (27,000 (10) compared to about 30,000). Yet in both cases the protein was

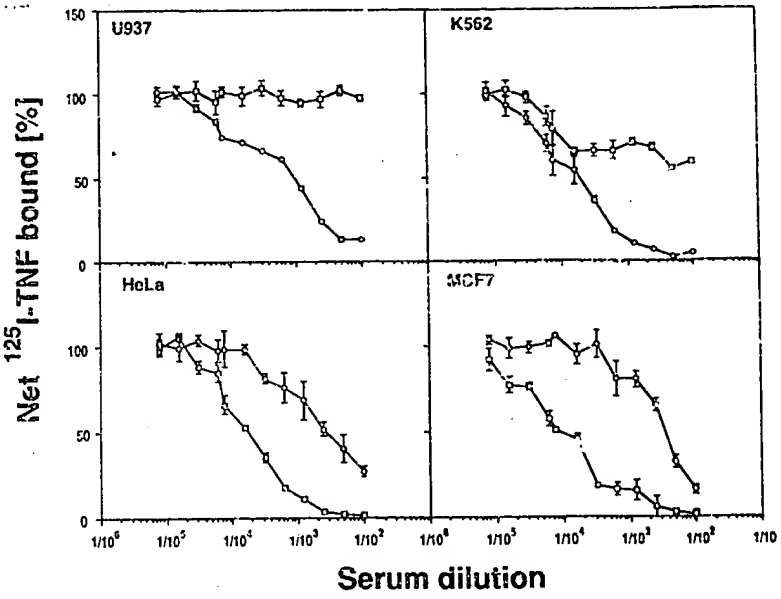

FIG. 6. **Inhibition of the binding of TNF to different cell lines with antisera to TBPI (○) and TBPII (□).** The net binding, observed in the absence of antisera (100%), was 2500 cpm in U937 cells, 1500 cpm in K562 cells, 2400 cpm in HeLa cells, and 1100 cpm in MCF7 cells.

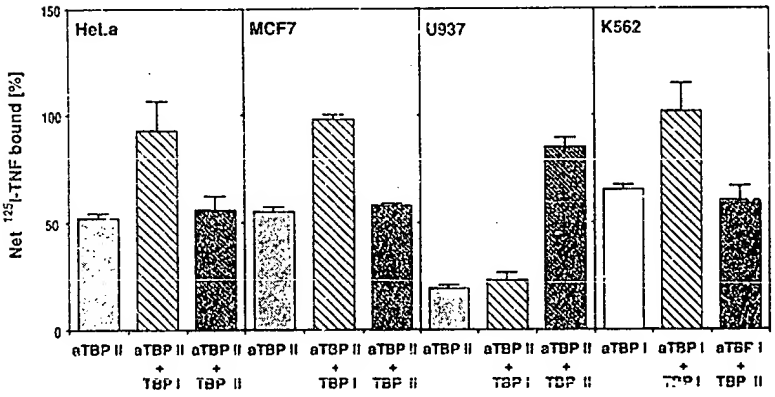

FIG. 7. **Contribution of cross-contamination between the antisera to TPPI and TBPII to their effects on the binding of TNF to HeLa, MCF7, U937, and K562 cells.** The antiserum to TBPI was applied at a dilution of 1:6000 and the antiserum to TBPII, at 1:400. The antisera were applied either alone or together with TBPI or TBPII at 1 μg/ml. For other details, see "Materials and Methods." The net binding observed in the absence of antiserum was 2800 cpm in HeLa cells, 1100 cpm in the MCF7 cells, 2300 cpm in the U937 cells, and 1200 cpm in the K562 cells.

eluted from a reversed phase HPLC column at the same acetonitrile concentration and in both the same NH_2-terminal amino acid sequence was observed. A likely explanation for the difference in molecular size is the higher probability for proteolytic degradation in the more lengthy manipulations involved in the chromatographic purification as compared to affinity purification.

The second protein which we purified from human urine (TBPII) resembles TBPI in its molecular size, its ability to bind TNF, and its inhibition of the cytocidal effect of TNF. However, TBPII can be clearly differentiated from TBPI by its lack of immunological cross-reactivity, differing NH_2-terminal amino sequences, and a difference in chromatographic properties which allowed its separation from TBPI by reverse phase HPLC. There are also indications that the two proteins are affected by proteases somewhat differently. As mentioned above, the molecular size of TBPI varies to some extent, depending on the purification procedure. This variation probably reflects degradation by proteases in the urine. However in spite of this variation in size the NH_2-terminal amino acid sequence of the protein remains unaltered implying that this degradation is restricted to its COOH terminus. On the other hand, TBPII does show signs of degradation at the NH_2 terminus, reflected in heterogeneity in the amino acid sequence.

Antibodies raised against the two urinary TNF-binding proteins inhibited the binding of TNF to cells. Consistent with recent evidence for existence of two species of TNF receptors whose relative proportion varies in different cell types,[2] antisera against the two proteins affected differentially the binding of TNF to different cells. These findings suggest immunological cross-reactivity and thus structural similarity between the urinary TNF-binding proteins and the cell surface receptors for TNF. Yet the molecular sizes of the soluble binding proteins are smaller than those reported for the cell surface receptors. The markedly differing extent to which TNF-α and TNF-β were bound by the soluble proteins, as opposed to the similar extent of binding of those cytokines by the membranous TNF receptors, is also indicative of some structural differences between these proteins. Detailed analysis of the amino acid sequences of the soluble and membranous TNF-binding proteins should provide further information as to their structural relationship.

Some idea as to the mechanisms which underlie the exist-

ence of TNF-binding proteins in both cell surface and soluble forms can be derived from studies on other proteins found in such dual forms. Several mechanisms for such a phenomenon have been observed, each implying a differing extent of interdependence between the formation of the soluble and the membranous forms of the protein. Soluble and membranous proteins which are structurally related, yet coded by different genes, and thus able to be formed independently of each other are known (*e.g.* Ref. 17). There are also membranous and soluble forms of proteins in which both forms of the protein are coded by the same gene and yet are synthesized independently by translation of different species of mRNA produced via alternative splicing pathways. The soluble and cell-associated forms of the immunoglobulins provide such an example. Yet soluble and membranous forms of the same protein can also be formed in a tightly coupled manner, the soluble protein being produced by cleavage or shedding of the membranous one (*e.g.* Refs. 18 and 19). Structural studies of the soluble form of the α-chain of the IL-2 receptor suggest that its formation involves such a step of proteolytic cleavage (20). Determining by what mechanism TBPI and TBPII are produced will contribute also to our understanding of the physiological function of these molecules. It is of particular interest to know whether the proteins are formed independently of the cell surface receptors or by their proteolytic cleavage, since in the latter case their formation should result in decreased amounts of the cellular receptors and thus may constitute a mechanism for a decrease in responsiveness of the cell to TNF.

Apart from the existence in the urine of what appears to be soluble forms of each of the two TNF receptors, probably derived from cells of different kinds, human urine has been found to contain also a soluble form of the α-chain of the IL-2 receptor (21). Furthermore, we could detect soluble receptors in urine for IL-6 and IFN-γ (22). There is probably a common underlying mechanism for the excretion of these different receptors. If this mechanism affects the receptors for other cytokines, it should be possible to isolate them also from urine. Indeed, there is limited evidence suggesting that receptors for several other cytokines are released by cells in a soluble form (*e.g.* 23–25). As our study shows, if these receptors are found to be excreted in the urine, their purification can be approached in a rather convenient way, simply by applying the crude urinary proteins on a column of the immobilized cytokine.

Another possibility raised by the findings in this study which may have practical importance is that receptors to cytokines, if presented to cells in a soluble form, may sequester the cytokine and thus serve as inhibitors. Detailed information on the structure of the receptors and knowledge of ways to produce them in a bioactive soluble form could provide us with inhibitory molecules which might act as therapeutic agents for suppressing overresponse to these cytokines in disease.

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MECHANISMS OF DISEASE

FRANKLIN H. EPSTEIN, M.D., *Editor*

CACHECTIN: MORE THAN A TUMOR NECROSIS FACTOR

BRUCE BEUTLER AND ANTHONY CERAMI

THE metabolic impact of infectious and neoplastic disease states has long been known to clinicians.[1-4] Invasive diseases may disrupt normal homeostatic mechanisms, both locally and systemically. For example, acute gram-negative infections frequently lead to profound metabolic acidosis and to biphasic changes in plasma glucose concentration, both seen in the context of hypotension, disseminated intravascular coagulation, and widespread tissue injury.[5-12] Chronic infectious diseases, as well as neoplastic diseases, may provoke a severe wasting diathesis, in which negative calorie and nitrogen balance lead to death despite the absence of a large parasite or tumor burden.

It was once widely believed that invasive agents were themselves responsible for these metabolic derangements. In recent years, however, there has been a growing awareness that endogenous mediators are essential elements in the pathogenesis of shock and cachexia alike.

The role of bacterial endotoxin in the pathogenesis of septic shock illustrates this principle. Endotoxin does not exert most of its effects on the host's metabolism directly, nor is it highly toxic to most mammalian tissues. On the contrary, endotoxin elicits the production of a host factor (or factors) that may in turn lead to shock and death. These factors appear to be produced by cells of hematopoietic origin. Transplantation studies in which marrow from endotoxin-sensitive (C3H/HeN) mice was infused into endotoxin-resistant (C3H/HeJ) recipients[13] have shown that sensitivity is conferred by cells of the donor. The macrophage has been suspected to be the cell responsible for endotoxin-induced injury and death, since various facultative intracellular bacteria, capable of eliciting reticuloendothelial hyperplasia, greatly enhance the endotoxin sensitivity of infected animals.[14,15] Moreover, endotoxin-induced macrophages produce, in vitro, a mediator capable of killing endotoxin-resistant mice.[16]

The host's response to endotoxin is perhaps an extreme illustration of its response to many pathogens. Identification of the mediators that confer endotoxin sensitivity would seem to be essential in the design of a specific strategy to arrest the development of shock in sepsis. Moreover, the agent (or agents) conferring endotoxin sensitivity may prove to be a mediator of general inflammation, which is important in the pathogenesis of many human diseases.

THE HISTORY OF CACHECTIN

"Cachectin" is the name applied to a macrophage hormone originally isolated in the course of studies aimed at delineating basic mechanisms of cachexia in chronic disease. In rabbits, trypanosomiasis produces a profound wasting diathesis.[17,18] Infected animals have a low parasite burden, yet become markedly anorectic and lose over half their body weight before dying of infection. Surprisingly, a striking lipemia develops during the final stages of the disease.[17] Rouzer and Cerami noted that the net elevation of plasma lipids was principally attributable to hypertriglyceridemia and that a clearing defect, caused by a systemic reduction in lipoprotein lipase activity, appeared to explain this phenomenon.[17]

Kawakami and Cerami[19] demonstrated that when endotoxin-sensitive (C3H/HeN) mice were given injections of bacterial lipopolysaccharide, systemic suppression of lipoprotein lipase activity and lipemia occurred. Endotoxin-resistant (C3H/HeJ) mice did not exhibit this response. However, lipemia and lipoprotein lipase suppression could be induced in endotoxin-resistant animals when they were injected with the serum of endotoxin-treated C3H/HeN mice. The cellular source of the factor was shown to be the macrophage. Macrophages obtained from sensitive (but not resistant) mice were able to produce a factor suppressing lipoprotein lipase when the cells were stimu-

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lated with lipopolysaccharide in vitro.[20] The factor responsible for this activity was named cachectin in recognition of its suspected role in the pathogenesis of cachexia.

Beutler et al.[21] purified cachectin to homogeneity and found it to be a polypeptide hormone with a subunit size of approximately 17 kilodaltons. Interestingly, cachectin was produced in considerable abundance, accounting for 1 to 2 percent of the total secretory product of endotoxin-activated RAW 264.7 cells,[21] a murine macrophage line. Similar quantities were later shown to be produced by mouse peritoneal macrophages. Very small amounts of the hormone are made by circulating monocytes,[22] unless the latter are "primed" with interferon-gamma, which greatly augments synthesis (unpublished data).

The hormone was shown to bind by means of a high-affinity receptor to adipocytes and myoblasts,[21] as well as a wide variety of other tissues.[23] After injection of endotoxin, cachectin appeared in the circulation within minutes, reached peak levels after two hours, and then rapidly declined in concentration.[23] The half-life of the hormone in the circulation was determined to be approximately six minutes.[23] Initial estimates of the net quantity of cachectin produced by a single animal in response to injection of endotoxin suggested that a rabbit might produce 1 mg of the hormone.[23] Abe and his coworkers[24] found cachectin or "tumor necrosis factor" (TNF) at a serum concentration of 0.3 μM in rabbits in shock. This would suggest that individual animals may actually produce several milligrams of cachectin in response to a lethal dose of lipopolysaccharide. Thus, the quantity of cachectin produced in such a case exceeds the amount required for lethal injury (see below).

The role of cachectin as a mediator of endotoxic shock was suggested by studies in which mice passively immunized against the hormone were found to be protected against the lethal effect of lipopolysaccharide.[25] With the availability of large quantities of recombinant cachectin, essentially free of contaminating endotoxin, it has been possible to examine the direct effects of the hormone in vivo and to determine whether cachectin itself could induce the shock and tissue injury associated with endotoxemia. When infused into rats in quantities similar to those produced endogenously in response to lipopolysaccharide, cachectin causes piloerection, diarrhea, and an ill, unkempt appearance. Typically, hemoconcentration, shock, metabolic acidosis, transient hyperglycemia followed by hypoglycemia, and hyperkalemia are observed. Severe end-organ damage is apparent on both gross examination and light microscopy.[26] The major arteries of the lungs become plugged with thrombi composed primarily of polymorphonuclear leukocytes, and a severe interstitial pneumonitis is present. Acute renal tubular necrosis, as well as ischemic and hemorrhagic lesions of the gastrointestinal tract, follows the administration of relatively low doses of the hormone (100 to 200 μg per kilogram of body weight in rats). In addition, adrenal and pancreatic hemorrhages are commonly noted.[26] Thus, cachectin evokes changes that essentially duplicate the pathologic effects of endotoxin administration.

Early in 1985, Beutler et al. noted that the amino terminal sequence of mouse cachectin was strongly homologous to that reported for human TNF.[27] It was also observed that cachectin and TNF possessed an identical spectrum of bioactivities and were immunologically indistinguishable, suggesting that they were in fact the same molecule.[27] This assumption was soon confirmed by genetic sequence analysis.[28]

The History of TNF

Among the diverse effects induced by endotoxin, few are so striking as the hemorrhagic necrosis of tumors, first noted nearly a century ago by Dr. William Coley, a New York City surgeon who observed the phenomenon in a patient with a sarcoma who contracted an intercurrent streptococcal infection.[29] Coley repeatedly administered bacterial broths, conditioned by the growth of serratia and streptococcus organisms, to patients with cancer in an attempt to induce hemorrhagic necrosis, with mixed results.[30,31] Shear and his colleagues[32-36] studied the phenomenon in animals and isolated the active agent (the "bacterial polysaccharide" — e.g., lipopolysaccharide) from cultures of serratia organisms.

The severe toxicity of lipopolysaccharide precluded its general use as an antineoplastic agent and stimulated a search for modified molecules capable of inducing hemorrhagic necrosis of tumors without causing shock, coagulopathy, and widespread end-organ damage. In 1962, O'Malley et al.[37] reported that serum obtained from mice in shock because of lipopolysaccharide infusion was capable of eliciting hemorrhagic necrosis of a transplantable tumor grown in another animal. A similar observation was made in 1975 in the laboratory of Dr. Lloyd Old.[38] A serum factor, derived from animals primed with bacillus Calmette Guérin and injected with endotoxin, was found to elicit hemorrhagic necrosis of transplantable tumors in vivo. Intensive studies of this molecule (TNF) were undertaken in the hope that it might present a means of avoiding the toxic effects of lipopolysaccharide while retaining the beneficial (tumorolytic) effect.

TNF was found to be a product of mononuclear phagocytes[39-42] and was observed in several different species.[40,42,43] Interestingly, the factor was found to be cytotoxic to selected tumor-cell lines (including the mouse fibrosarcoma line L-929) in vitro. The L-929 cell cytotoxicity assay allowed purification of TNF. This was achieved by Dr. Bharat Aggarwal and his colleagues,[44] who also succeeded in purifying lymphotoxin, a tumorolytic protein derived from lymphocytes.[45]

The lymphotoxin and TNF genes appear to be products of an ancient tandem duplication event.[46] Both genes are closely linked on human chromosome 6 and, as such, are HLA-linked.[46] In the mouse, the

genes lie within the D region of the H-2 complex, approximately 70 kilobases proximal to the D gene (Fischer-Lindahl K: personal communication). The proteins specified by these genes share approximately 30 percent homology at the amino acid level.[46-48] In addition, they have a highly concordant range of biologic activities and appear to bind to a common receptor.[49] However, lymphotoxin is produced only by T lymphocytes[50] and B lymphoblastoid cell lines[45,51] in response to mitogenic or specific antigenic stimuli. TNF, as noted above, is a product of macrophages, is produced in greatest quantity after exposure to lipopolysaccharide, and is then produced in far greater abundance than lymphotoxin.

Clinical trials of TNF as an antineoplastic agent are currently in progress. No consensus has emerged about the efficacy of the agent. Since TNF (cachectin) is a primary mediator of the lethal effect of endotoxin, it would seem that the therapeutic index of TNF in patients with cancer might differ little from that of lipopolysaccharide.

Thus, two very different lines of investigation — one aimed at isolating an endogenous mediator of tumor necrosis, and another aimed at isolating endogenous mediators of cachexia and shock — led to the realization that both tumor necrosis and endotoxic shock arise through the action of the same macrophage hormone, cachectin/TNF.

The Structure of Cachectin/TNF and the Regulation of Cachectin Expression

The primary structure of cachectin/TNF derived from three mammalian species (rabbits,[52,53] mice,[28,54,55] and humans[47,56]) has now been determined. In each species, cachectin is produced as a prohormone, which appears to be biologically inactive in in vitro assays of lipoprotein lipase suppression and cytotoxicity (unpublished data). The propeptide, which like cachectin itself is extensively conserved, is cleaved at several sites to yield the mature polypeptide.[57] The human propeptide contains 76 additional amino acids at the amino terminal end of the molecule. It is not known at present whether this sequence (or its cleavage products) exhibits a biologic activity responsible for its extensive conservation.

In each species, two cysteine residues are present, apparently connected by a disulfide bridge.[44] This arrangement is lacking in lymphotoxin, which possesses but a single cysteine residue. Nonetheless, it is thought that the two cytokines have retained conformational similarity, since they compete for binding to a common receptor.

Studies of the control of cachectin biosynthesis[58] have suggested that hormone production is tightly regulated. Both transcriptional and post-transcriptional activation must occur to allow its production.[58] Thus, the chances of inadvertent release of cachectin are minimized.

Glucocorticoid hormones, which strongly antagonize the effects of endotoxin if they are administered before infectious or endotoxic insult,[58] will completely inhibit cachectin biosynthesis, both by diminishing the quantity of cachectin mRNA that is produced in response to lipopolysaccharide and by preventing its translation. Glucocorticoids are effective in preventing cachectin biosynthesis only if they are applied to macrophages in advance of activation. If dexamethasone is added to macrophage cultures after activation by lipopolysaccharide, no inhibiting effect occurs. Thus, the difficulty encountered in mitigating the effects of sepsis by administration of steroids is understandable in molecular terms. Inhibition of cachectin biosynthesis by steroids does not occur once widespread reticuloendothelial induction has been initiated.

The 3′-untranslated region of the cachectin mRNA molecule contains a sequence element composed entirely of A and U (adenosine and uridine) residues, arranged as repeating octameric units [(UUAUUUAU)n].[28] Comparison of human and murine cachectin mRNA reveals that this sequence is conserved in toto over a span of 33 nucleotides, suggesting that it may have a discrete regulatory function. In addition, mRNA molecules encoding other inflammatory mediators, including lymphotoxin, interleukin-1, granulocyte-macrophage colony-stimulating factor (GM-CSF), and most of the interferons (representing all subclasses) also contain UUAUUUAU sequences of varying length in the 3′-untranslated region.[28] Recently, Shaw and Kamen[59] demonstrated that modified beta-globin mRNA molecules, containing the GM-CSF consensus sequence, are markedly destabilized in vivo. The modified globin mRNA is also strongly superinducible; in the presence of cycloheximide, greatly elevated levels of the message are expressed. Thus, it would seem that this sequence is probably involved in control of cachectin-gene expression, acting at a post-transcriptional level.[28,56]

Mechanism of Action

The postreceptor effects of cachectin are known only in general terms. Torti et al.[60] showed that cachectin acts to suppress biosynthesis of several adipocyte-specific mRNA molecules and to prevent morphologic differentiation of pre-adipocytes. Presumably, lipoprotein lipase is one of many enzymes specifically suppressed at a transcriptional level by the action of this hormone. Cachectin also acts to induce the biosynthesis or release (or both) of specific proteins, including Class I major histocompatibility antigen,[61] GM-CSF,[62] and interleukin-1.[63,64] These biochemical effects of cachectin may explain some, but probably not all, of its effects as observed at the level of tissues and organ systems.

The precise mechanism by which cachectin elicits shock and the mechanism by which it induces hemorrhagic necrosis remain unknown. It is likely that the two effects are inextricably related.

As early as 1952, Algire et al.[65] studied the histologic appearance of the necrotizing reaction that occurred within a tumor mass after injection of lipopoly-

saccharide, and concluded that the injury was related to diminished tissue perfusion.[65] The tumor-necrotizing effect of lipopolysaccharide may be duplicated by mechanical obstruction of regional blood flow.[65] Moreover, tumors exhibit site-dependent sensitivity to the effects of TNF in vivo. Dermal implants of tumor may be far more liable to destruction than visceral implants (Fidler I: personal communication). Hemorrhagic necrosis appears to arise through vascular events and may be mechanistically unrelated to tumorolysis in vitro.

Recent studies have suggested that cachectin alters the hemostatic properties of the vascular endothelium, inducing the production of a procoagulant activity,[66,67] and inhibiting the expression of thrombomodulin at the cell surface.[66] Both these effects might be expected to favor the accretion of thrombi, leading to disseminated intravascular coagulation at a systemic level and to occlusion of tumor vessels. Among other effects of cachectin on endothelial cells, altered patterns of antigenic expression,[61,68-70] interleukin-1 production,[64,71] and cellular rearrangement[72] in vitro have been observed. It appears that cachectin is directly toxic to vascular endothelial cells.[73] The sum of these effects may explain many of the actions of the hormone in vivo.

It is likely that various vascular beds respond to cachectin in different ways and that certain tumor vessels are more sensitive to the effects of cachectin than the vessels of most normal tissues. It is also quite clear that hemorrhagic necrosis may occur in normal tissues obtained from animals treated with sublethal doses of cachectin.[26] In rats, for example, the cecum is invariably infarcted after administration of cachectin at a dose in excess of 600 μg per kilogram.[26]

Cachectin is most probably a proximal mediator of the effects of lipopolysaccharide, which acts to initiate a large number of events leading to shock and tissue injury. Leukotrienes[74] and platelet-activating factor[75-77] have repeatedly been implicated in the pathogenesis of endotoxic shock, and it is quite possible that cachectin triggers their production. The infiltration of polymorphonuclear leukocytes into numerous organs, particularly the lungs, after cachectin administration may result in part from elaboration of these secondary mediators.

Fluid and electrolyte sequestration invariably accompany (and contribute to) endotoxic shock. Hemoconcentration is observed soon after the administration of cachectin and presumably reflects a rapid decline in intravascular volume. This is most likely attributable to an endothelial lesion, which allows the escape of plasma water and electrolytes to the extravascular space.

The "third space" into which plasma water and electrolytes are ultimately deposited in endotoxic shock is most probably the intracellular compartment. Cachectin has recently been shown to diminish the transmembrane potential of muscle cells.[78] This phenomenon may reflect sodium permeabilization for which the cells cannot compensate or inefficiency of the sodium–potassium-dependent ATPase responsible for maintenance of the electrochemical gradient; either of these conditions would lead to intracellular volume expansion.

In addition to exerting this direct effect, cachectin is known to induce release of interleukin-1 by monocytes and endothelial cells.[63,64] Interleukin-1, in turn, may elicit some of the features that characterize endotoxin poisoning (Dinarello C: personal communication), contributing to the fever, hypotension, neutropenia, and thrombocytopenia that prevail.

The mechanism through which lysis of certain transformed cells occurs in vitro remains an intriguing problem. This phenomenon, once understood in biochemical terms, may conceivably lead to the design of a novel and effective chemotherapeutic strategy.

Cachectin as an Inflammatory Mediator

Cachectin was isolated as a hormone capable of suppressing the expression of lipoprotein lipase and therefore capable of preventing the uptake and storage of exogenous triglyceride. It was found to suppress the expression of several other adipose-specific enzymes,[60] to inhibit the uptake of acetate by fat,[79] and to cause a net loss of triglyceride from fat ("cachexia in vitro").[60] The administration of recombinant cachectin to laboratory animals causes anorexia and weight loss (unpublished data). However, the role of cachectin in the cachexia of chronic diseases remains to be determined, since cachectin cannot be detected in the plasma of cachectic patients. This finding may reflect the relatively low sensitivity of immunoassays that are currently available.

Cachectin (TNF) has also been considered by some as an endogenous antineoplastic agent[80] — e.g., as a potential mechanism of immune surveillance. It is not clear, however, that any tumors can actually induce cachectin biosynthesis, and it is quite clear that most tumors are highly resistant to the hormone's cytotoxic effect.

Irrespective of the roles in which it was originally cast, cachectin has emerged as a mediator of general inflammation, and a variety of recent observations suggest that the molecule may play an important part in diverse human disease processes. Cachectin is an endogenous pyrogen, capable of inducing fever both through a direct effect on hypothalamic neurons and through the peripheral induction of interleukin-1,[63] which in turn elicits fever. Hence, administration of lipopolysaccharide-free preparations of cachectin to rabbits evokes a biphasic febrile response.[63] The initial rise in temperature is attributable to the direct effect of the hormone, whereas the second rise results from interleukin-1 release.

Cachectin also exhibits osteoclast-activating factor activity.[81] In this respect, it again bears a resemblance to interleukin-1, which was earlier shown to possess osteoclast-activating factor activity.[81] And like interleukin-1, cachectin is capable of stimulating synovial-cell production of prostaglandin E_2 and collagenase.[82]

Cachectin activates polymorphonuclear leukocytes, stimulating their adhesion to endothelial-cell surfaces and enhancing their phagocytic activity.[83,84] A separate effect of the hormone on the endothelial cells themselves also promotes neutrophil adhesion.[83] In vitro these actions undoubtedly reflect the histopathologic changes evoked by cachectin produced in vivo.

Recently, it has also been reported that cachectin (TNF) induces the differentiation of certain myeloid cell lines in vitro.[85,86] In addition, it has been noted that cachectin can induce GM-CSF production by a variety of cell types.[62] However, it has also been noted that cachectin can act to inhibit hematopoiesis directly in in vitro assay systems, decreasing the expression of granulocyte–macrophage colony-forming units,[87,88] erythroid burst-forming units,[88] and granulocyte–erythroid–macrophage–megakaryocyte-forming units.[88] The net effect of the hormone on hematopoiesis in vivo has not been described.

Numerous inflammatory disorders of diverse origins may depend on the production of cachectin, with all its attendant consequences. For example, excessive production of collagenase and prostaglandin E_2 production may lead to the loss of bone and cartilage in rheumatoid arthritis; this entire process may depend in part on the production of cachectin at a local level. Similarly, inflammatory diseases of the central nervous system, gastrointestinal tract, lungs, kidneys, and other tissues may depend on cachectin release. In years to come, a wide variety of inflammatory diseases will undoubtedly be studied in order to determine whether cachectin, produced autonomously or in response to a specific pathogenic stimulus, has an important pathogenic role.

The range of stimuli known to evoke cachectin production is incompletely known at present. Endotoxin remains the most potent stimulus known. However, virus particles[89,90] and certain other biologic agents[91] also trigger cachectin production. With additional study, it seems likely that many infectious agents will be identified as inducers.

It would seem appropriate to ask what beneficial function of cachectin has justified its evolutionary conservation. Recently, it has been shown that sublethal quantities are capable of protecting mice from challenge with an otherwise lethal inoculum of *Plasmodium berghei* (Schofield L: personal communication). It has also been shown that the hormone exerts an antiviral effect in vitro under certain circumstances.[92,93] In addition, C3H/HeJ mice, which cannot produce cachectin[58] because they have a genetic lesion,[94] are far more susceptible to gram-negative infections than normal mice are.[95] Thus, certain infections may be controlled effectively through the action of this and related cytokines.

Relation of Cachectin to Other Hormonal Factors

Through "priming" (e.g., infecting animals with facultative intracellular bacteria such as BCG or *Corynebacterium parvum*), greatly augmented production of cachectin[38,40,96,97] may be achieved in vivo after lipopolysaccharide administration. Although this phenomenon remains incompletely understood, it is clear that cachectin production is strongly influenced by other mediators.

Interferon-gamma, which has been shown to activate macrophages, thereby enhancing tumoricidal activity,[98-100] production of hydrogen peroxide,[100,101] phagocytic potential,[100-103] and other defensive functions,[100] also augments the production of cachectin in response to endotoxin.[22,104] Interferon-gamma appears to achieve this effect by enhancing lipopolysaccharide-induced cachectin-gene transcription and cachectin-mRNA translation.[104] It is likely that interferon-gamma (or a related cytokine) contributes to the priming phenomenon.

As previously mentioned, glucocorticoid hormones strongly inhibit cachectin production. The inhibiting effect may be demonstrated in vitro in the presence of cortisol concentrations corresponding to normal human free cortisol levels in vivo. Thus, glucocorticoid hormones may prevent unconstrained release of cachectin, with all its deleterious consequences, which might otherwise occur in the presence of a relatively minor infection. The greatly increased susceptibility to infection of adrenalectomized or otherwise glucocorticoid-deficient patients may reflect the loss of this control mechanism.

Antagonism of Cachectin Action in the Management of Inflammatory Disorders

Therapeutic measures aimed at attenuating the inflammatory response are at present very nonspecific. Glucocorticoid hormones and a variety of cytotoxic drugs can effectively impede inflammation, but also affect host metabolism and arrest the proliferation of many normal host tissues. As a central mediator of inflammation, cachectin is an isolated target for pharmacotherapeutic intervention.

It has been demonstrated that mice treated with a polyclonal antiserum directed against mouse cachectin become resistant to the lethal effect of lipopolysaccharide.[25] Thus, it would seem possible that neutralizing monoclonal antibodies directed against human cachectin may prove to be useful in the treatment of sepsis, particularly in its early stages.

It remains to be seen whether such antibodies (or other cachectin antagonists) will also prove useful in the treatment of other pathologic states in which inflammation has a role. However, it is anticipated that specific neutralization of cachectin and of related cytokines may offer new therapeutic directives with which to treat a broad spectrum of diseases.

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Cytotoxins (Tumour Necrosis Factor, Lymphotoxin and Others): Molecular and Functional Characteristics and Interactions with Interferons

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ABBREVIATIONS

The following abbreviations and synonyms will be used

ADCC = antibody-dependent cytotoxicity
CHI = cycloheximide
CTX = cytotoxins
DTH = delayed-type hypersensitivity
HA = haemagglutination units
IFN = interferon
IL-1 = interleukin 1
LPS = bacterial lypopolysaccharides
LT = lymphotoxin
mAb = monoclonal antibody
MHC = major histocompatibility complex
MP = mononuclear phagocytes

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NK = natural killer
NKCF = natural killer cytotoxic factor
PAGE = polyacrylamide gel electrophoresis
PBMC = peripheral-blood mononuclear leukocytes
PHA = phytohaemagglutinin
PMA = 4βphorbol-12-myristate-13-acetate
SDS = sodium dodecyl sulphate
TNF = tumour necrosis factor
VSV = vesicular stomatitis virus

I INTRODUCTION

Awareness of the anticellular activity of the interferons (IFNs), and of the existence of cytokines other than IFNs with anticellular activity, has developed in parallel. Initial studies on the anticellular effects of IFNs were with crude preparations (Pauker *et al.*, 1962). This raised doubts about whether IFN in these preparations did indeed mediate the observed effects, and not other, contaminating, cytokines. Conversely, the existence of cytokines, other than IFNs, with cytotoxic activity ('cytotoxins'—CTXs) has for quite a time been questioned in view of the fact that the crude preparations in which these mediators were detected. (Granger and Kolb, 1968; Ruddle and Waksman, 1968; Carswell *et al.*, 1975) did contain IFNs. With the purification of IFNs and, very recently, two CTXs—lymphotoxin and tumour necrosis factor (Aggarwal *et al.*, 1984, 1985; Haranaka *et al.*, 1985) it was confirmed that both the IFNs and the CTXs have their own anticellular activities. It was also observed that the two kinds of mediators may act in concert: their anticellular activities are synergistic; other effects of CTXs, besides their anticellular ones, are also potentiated by IFNs. Furthermore, IFNs promote the formation of CTXs, and CTXs can induce the formation of IFNs.

We are attempting now to throw light on the mechanisms which underlie these interrelationships of IFNs and CTXs and to understand their physiological significance. Our concepts on these subjects are still in a state of flux. In fact, even the terminology for the CTXs is still undefined and may have to remain so until we have a better idea of the extent of multiplicity and heterogeneity of the CTXs. In this review, I have tried to extract from the fragmentary information available as comprehensive an understanding as possible and to speculate on aspects in which there are still gaps in our knowledge. A suggestion made by Donald Metcalf (1975) may be appropriate to apply at this point: 'Remember that no matter how enthusiastic you may be about the significance of your observations, if your paper is re-read in 10 years' time, your original interpretation of the data will almost certainly be wrong, if not hilariously so.' It may take much less than 10 years in the field of CTX research.

This review focuses on those aspects of our knowledge of CTXs concerning the interrelationship between CTXs and IFNs. For a survey of other aspects

of CTX research there are a number of r
Klostergaard *et al.*, 1981; Ruddle and Clifford

II THE CYTOT

A Terminology

There is at present some ambiguity in the n
coined in the past for crude preparations of
the purified proteins (Table I). Yet cytotoxic
tions cannot always be fully accounted for
bearing the same name. Furthermore, we no
overlap in the kinds of CTXs present in cru
differing names. To avoid misunderstanding
review to all crude preparations of the pro
toxins, specifying when necessary the conditi
is the term 'NKCF' which will be used here
CTXs produced by NK cells in response t
photoxin' and 'TNF' will be applied to
proteins.

Nevertheless, it may be useful to review
the crude CTX preparations formerly in us
of the major milestones in CTX research. T
coined for CTX preparations induced in lu
lectins and by antigens. These were first a
(1968) and by Ruddle and Waksman (19
G. Granger and his collaborators demons
chemical as well as in serological properties
fractions of such preparations separated
exchange chromatography, have been re
appeared to be main size populations of the
tion of LT-α in DEAE-cellulose chromatog
et al., 1978).

Production of some cytotoxic factors
phagocytes (MP) was noted quite some time
the first detection of that specific CTX we
(TNF) can be traced to a study in 19
necrosis in a transplantable methylcholanth
produced CTXs. These CTXs were induced
lipopolysaccharides (LPS) following prime
(Carswell *et al.*, 1975). Initially circumstan

of CTX research there are a number of useful reviews (Rosenau, 1980; Klostergaard *et al.*, 1981; Ruff and Gifford, 1981a; Ruddle *et al.*, 1983).

II THE CYTOTOXINS

A Terminology

There is at present some ambiguity in the terminology of the CTXs. Terms coined in the past for crude preparations of CTX have now been adopted for the purified proteins (Table I). Yet cytotoxic activities of the crude preparations cannot always be fully accounted for by those of the isolated proteins bearing the same name. Furthermore, we now know that there is a significant overlap in the kinds of CTXs present in crude preparations that were given differing names. To avoid misunderstanding, I have chosen to refer in this review to all crude preparations of the proteins by the general term cytotoxins, specifying when necessary the conditions of induction. (An exception is the term 'NKCF' which will be used here, as elsewhere, for describing the CTXs produced by NK cells in response to tumor cells.) The terms 'lymphotoxin' and 'TNF' will be applied solely in reference to the purified proteins.

Nevertheless, it may be useful to review briefly the various terms for the crude CTX preparations formerly in use, as a way of introducing some of the major milestones in CTX research. The term 'lymphotoxin' (LT) was coined for CTX preparations induced in lymphocyte cultures by mitogenic lectins and by antigens. These were first described by Granger and Kolb (1968) and by Ruddle and Waksman (1968). Later studies, primarily by G. Granger and his collaborators, demonstrated heterogeneity in physiochemical as well as in serological properties of PHA-induced CTXs. Differing fractions of such preparations, separated by gel filtration and by ion-exchange chromatography, have been termed 'LT-α, β and γ' for what appeared to be main size populations of the CTXs. LT-α1 denoted a subfraction of LT-α in DEAE-cellulose chromatography, and so on (*c.f.* Granger *et al.*, 1978).

Production of some cytotoxic factors also by cultured mononuclear phagocytes (MP) was noted quite some time ago (*cf.* Pincus, 1967). However, the first detection of that specific CTX we now call 'tumor necrosis factor' (TNF) can be traced to a study in 1975, describing induction of haemorrhagic necrosis in a transplantable methylcholanthrene-induced sarcoma by *in vivo* produced CTXs. These CTXs were induced by injecting mice with bacterial lipopolysaccharides (LPS) following priming with Bacillus-Calmette Guérin (Carswell *et al.*, 1975). Initially circumstantial evidence, and later serological

TABLE I Modes of Induction, Terms Used for Differing Preparations of CTXs and Identity of Concomitantly Formed IFNs

Producer cells	Inducing agents	Terms for crude preparations of induced CTXs	Terms for isolated CTXs	IFNs induced
T-cells (primarily Lyt-1$^+$)	T-cell mitogens (antigens; lectins; allogeneic cells; IL-2 . . .)	Lymphotoxins (LTs)	LT (or TNF-β) (and TNF-α?)	IFN-γ
Lymphoblastoid B cells	4β-phorbol-12-myristate-13-acetate (PMA)			
Mononuclear phagocytes	LPS; PMA; Sendai virus; Tumour cells.	Tumour necrosis factor (TNF)	TNF (or "Necrosin" or TNF-α or "cachectin")	IFN-α and β
NK cells	Tumour cells	Natural killer cytotoxic factor (NKCF)	(TNF ?)	IFN-α

examination (Männel *et al.*, 1980). [illegible] produced *in vivo* with those produced [illegible] phagocytes (MP).

More recent studies have demonstrated [illegible] natural-killer (NK) cells, in response to [illegible] CTX preparations were termed 'natural [illegible] (Wright and Bonavida, 1982).

B. Characteristics of the Isolated CTXs

Crude preparations of lymphotoxins, tumour [illegible] contain IFNs. Since IFNs by themselves can [illegible] activities, separating the two kinds of factors [illegible] functional criteria whereby they differ from [illegible] difference between the two is that CTXs [illegible] characterizes the IFNs. In addition, clear differences [illegible] the way IFNs and CTXs exert their antiviral [illegible] IFNs, like all other effects of the IFNs, is dependent [illegible] proteins in the affected cell. On the other hand, [illegible] to be mediated independently of protein synthesis [illegible] potentiated by inhibitors of protein and [illegible] species-specificity to the extent observed in IFNs [illegible] the cytotoxic function of CTX preparations.

As shown in Fig. 1, which demonstrates [illegible] from an IFN (IFN-γ) and separation of the [illegible] these functional differences between CTXs and [illegible] detection of the one in the presence of the other. [illegible] the presence of CTXs by measuring the antiviral [illegible] CTXs can be specifically determined in the presence [illegible] cytotoxic effects exerted in the presence of [illegible] synthesis. Alternatively, the cytotoxic activity [illegible] determined in the presence of IFNs in assay [illegible] which would not respond to the species-specific [illegible] 1929 cells for detecting human TNF).

Two CTXs have recently been isolated [illegible] about 25 Kd was isolated from preparations [illegible] B-lymphoblastoid cell line (Aggarwal *et al.*, [illegible] glycosylated protein, with an M_r of about [illegible] CTXs induced in cells of a promyelocytic [illegible] Haranaka *et al.*, 1985). The two proteins [illegible] structure, so that while initially they were [illegible] tumour necrosis factor (TNF), it was [illegible]

examination (Männel *et al.*, 1980), affirmed the identity of these CTXs produced *in vivo* with those produced *in vitro* by cultured mononuclear phagocytes (MP).

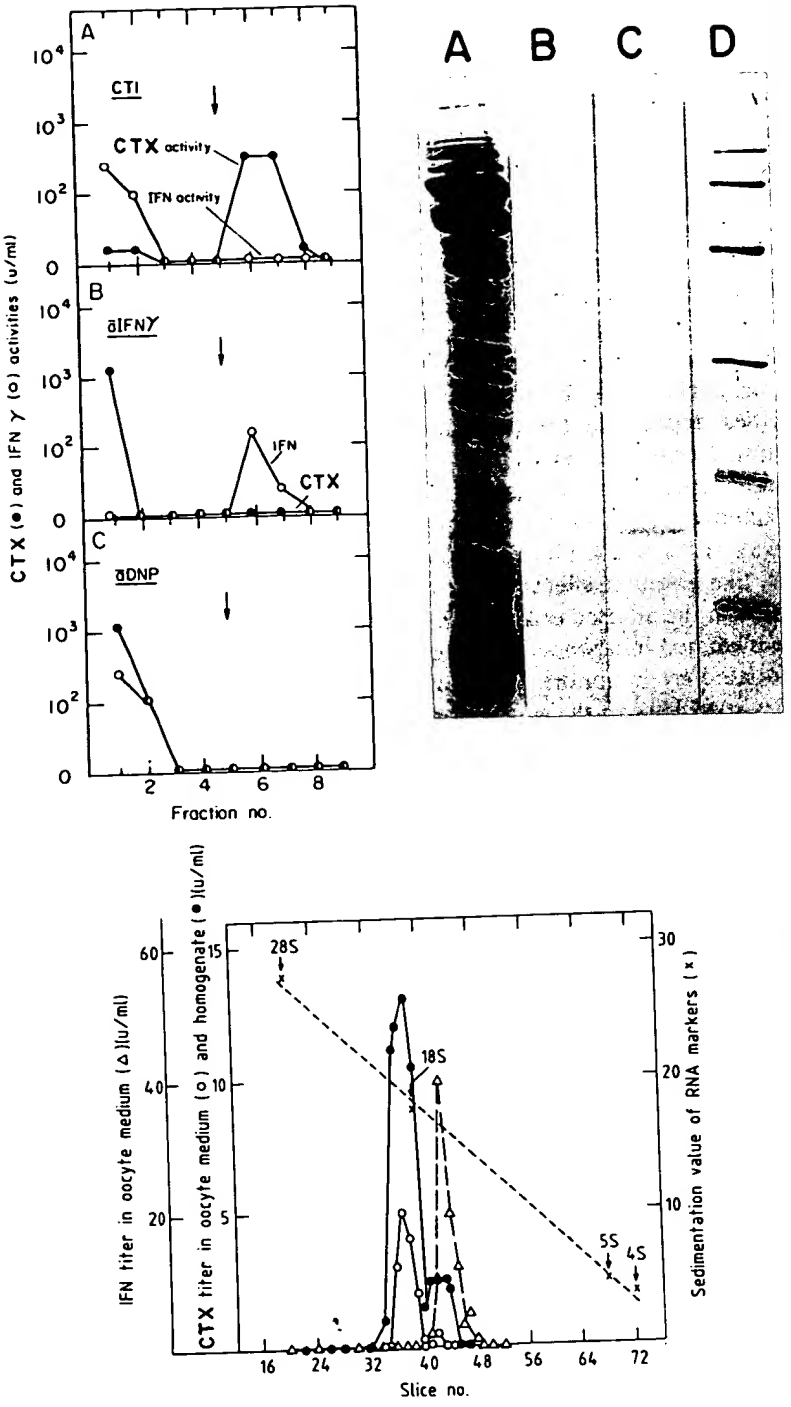
More recent studies have demonstrated production of CTXs also by natural-killer (NK) cells, in response to certain tumour target cells; those CTX preparations were termed 'natural killer cytotoxic factors' (NKCF) (Wright and Bonavida, 1982).

B Characteristics of the Isolated CTXs.

Crude preparations of lymphotoxins, tumour necrosis factor and NKCF all contain IFNs. Since IFNs by themselves can exert cytostatic and cytotoxic activities, separating the two kinds of factors depends on first defining functional criteria whereby they differ from each other. The most evident difference between the two is that CTXs do not have the antiviral activity that characterizes the IFNs. In addition, clear differences can also be discerned in the way IFNs and CTXs exert their anticellular effects: killing of cells by IFNs, like all other effects of the IFNs, is dependent on synthesis of some proteins in the affected cell. On the other hand, cell killing by CTXs is found to be mediated independently of protein synthesis and, in fact, to be greatly potentiated by inhibitors of protein and of RNA synthesis. Furthermore, species-specificity to the extent observed in IFN function is not observed in the cytotoxic function of CTX preparations.

As shown in Fig. 1, which demonstrates separation of a CTX (TNF) from an IFN (IFN-γ) and separation of the mRNAs for the two proteins, these functional differences between CTXs and IFNs can allow specific detection of the one in the presence of the other. IFNs can be determined in the presence of CTXs by measuring the antiviral effect of the former, while CTXs can be specifically determined in the presence of IFN by measuring cytotoxic effects exerted in the presence of inhibitors of RNA or of protein synthesis. Alternatively, the cytotoxic activity of CTXs can be specifically determined in the presence of IFNs by using cells of a remote animal species which would not respond to the species-specific IFN effect (e.g. use of mouse L929 cells for detecting human TNF).

Two CTXs have recently been isolated—one, a glycoprotein with an M_r of about 25 Kd was isolated from preparations of CTXs produced by cells of a B-lymphoblastoid cell line (Aggarwal *et al.*, 1984) and the other, a non-glycosylated protein, with an M_r of about 17 Kd—from preparations of the CTXs induced in cells of a promyelocytic cell line (Aggarwal *et al.*, 1985a; Haranaka *et al.*, 1985). The two proteins turned out to be closely related in structure, so that while initially they were called lymphotoxin (LT) and tumour necrosis factor (TNF), it was suggested recently that they be named



TNF β and α respectively. The mouse homolog 'necrosin' (Kull and Cuatrecasas, 1984). This (1943), for an *in vivo*, induced factor, recovered injected with turpentine, that could induce cel bling that observed in inflammation.

The amino acid sequence of LT and TNF, a the proteins themselves as well as from sequen genomic DNAs, were found to be remarkably 1984, 1985; Gray *et al.*, 1984; Pennica *et al.*, *et al.*, 1985; Fransen *et al.*, 1985). By introduci the two sequences can be aligned with an identi (including conservative changes) of 46% of the largely confined to a single region in the mole and 86–118 in LT). That 32-amino-acid regi disulfide bridge (between cysteins 69 and 10 this sequence a loop structure separated from

The similarity in structure between TNF functions which, so far, appear to be indistu ferences in structure between the two prote functionally meaningful. The sequence homo significantly less than that between human an 1985; Marmenout *et al.*, 1985; Pennica *et al.*, logy (including conservative changes) in the from the two sources is observed not only th but also in the structure of their leader portio longer (76 residues) than in LT (34 residues) from those coding for most of the mature pro

Fig. 1. (A) and (B) Isolation of TNF by the use of a mi (Hahn *et al.*, 1985). (C) Dissociation of the mRNAs microinjection technique for determining translational *et al.*, 1984).

(A) Fractionation of a cytokine preparation contai adsorbents constructed with mAbs against TNF (A1). proteins were eluted with 0.2 M NH_4OH (arrow).

(B) SDS/15%-PAGE of TNF purified by immunoad protein (A); NH_4OH-eluted fraction from the immun dinitrophenyl mAb (B); TNF purified on the mAb ag standards (94; 67; 43; 30; 20.1 and 14.4) (D).

(C) 1.2% agarose–methylmercuric hydroxide gel e stimulated human PBMC. RNA extracted from slices o oocytes and the level of cytotoxic activity in the oocyte (○) and IFN activity (Δ) in the oocyte incubation me microinjection.

TNF activity was determined by measuring cytoto of CHI and IFN-activity by determining reduction in th virus (VSV) on WISH cells.

TNF β and α respectively. The mouse homologue of TNF was initially called 'necrosin' (Kull and Cuatrecasas, 1984). This term was coined by Menkin (1943), for an *in vivo*, induced factor, recovered from pleural exudates of dogs injected with turpentine, that could induce cellular injury in animals resembling that observed in inflammation.

The amino acid sequence of LT and TNF, as established from analysis of the proteins themselves as well as from sequencing their complementary and genomic DNAs, were found to be remarkably homologous (Aggarwal *et al.*, 1984, 1985; Gray *et al.*, 1984; Pennica *et al.*, 1984; Shirai *et al.*, 1985; Wang *et al.*, 1985; Fransen *et al.*, 1985). By introducing gaps in the structure of LT, the two sequences can be aligned with an identity of 28% and with homology (including conservative changes) of 46% of their amino acids. Differences are largely confined to a single region in the molecules (residues 69–101 in TNF and 86–118 in LT). That 32-amino-acid region is, in TNF, confined by a disulfide bridge (between cysteins 69 and 101) which probably imposes on this sequence a loop structure separated from the rest of the molecule.

The similarity in structure between TNF and LT is reflected in their functions which, so far, appear to be indistinguishable. However, the differences in structure between the two proteins may yet be found to be functionally meaningful. The sequence homology between TNF and LT is significantly less than that between human and mouse TNF (Fransen *et al.*, 1985; Marmenout *et al.*, 1985; Pennica *et al.*, 1985). Almost complete homology (including conservative changes) in the amino acid sequences of TNF from the two sources is observed not only throughout the mature molecules but also in the structure of their leader portion, which in TNF is significantly longer (76 residues) than in LT (34 residues): it is encoded by exons distinct from those coding for most of the mature protein. The effective preservation,

Fig. 1. (A) and (B) Isolation of TNF by the use of a monoclonal antibody (mAb) to the protein (Hähn *et al.*, 1985). (C) Dissociation of the mRNAs for TNF and IFN-γ using the oocyte microinjection technique for determining translational activity of the two mRNAs (Wallach *et al.*, 1984).

(A) Fractionation of a cytokine preparation containing the two proteins using immunoadsorbents constructed with mAbs against TNF (A), IFN-γ (B) or dinitrophenyl (C). Bound proteins were eluted with 0.2 M NH_4OH (arrow).

(B) SDS/15%-PAGE of TNF purified by immunoadsorption. The crude preparation of the protein (A); NH_4OH-eluted fraction from the immunoadsorbent constructed with the anti-dinitrophenyl mAb (B); TNF purified on the mAb against this protein (C); molecular weight standards (94; 67; 43; 30; 20.1 and 14.4) (D).

(C) 1.2% agarose–methylmercuric hydroxide gel electrophoresis of poly(A)$^+$ RNA from stimulated human PBMC. RNA extracted from slices of the gel was microinjected into *Xenopus* oocytes and the level of cytotoxic activity in the oocyte homogenates (●) as well as the cytotoxic (○) and IFN activity (△) in the oocyte incubation media were determined 24 hours following microinjection.

TNF activity was determined by measuring cytotoxic effect on SV-80 cells in the presence of CHI and IFN-activity by determining reduction in the cytophatic effect of Vesicular stomatitis virus (VSV) on WISH cells.

Sedimentation value of RNA markers (×)

through evolution, of parts of TNF differing from LT implies that these sequences are functionally important and that their alteration in the evolution of LT may be functionally meaningful.

At present it is not clear how many other CTXs there are besides TNF and LT. The recently described 'perphorins' are CTXs that appear to function quite differently from LT and TNF and are therefore likely to differ from these proteins in structure as well. The perphorins are components of cytoplasmic granules of cytotoxic T-lymphocytes and of NK cells. Upon interaction of the isolated granules or of the intact cytotoxic cells with membranes the proteins form, in a Ca^{2+}-dependent manner, tubular complexes that are inserted into the membranes and cause cytolysis (Podack (1985)). Purification of "perphorin" and initial characterization of its molecular properties have recently been reported (Young *et al.*, 1986).

There have been a few reports of purification of some other CTXs that apparently differ from TNF and LT (e.g. Ransom *et al.*, 1985; Rubin *et al.*, 1985). Moreover, some proteins of non leukocyte origin which exert cytostatic effects have also been isolated (e.g. cf. Roberts *et al.*, 1985). It is not yet known to what extent, if any, these proteins are structurally related to TNF and LT.

III CTX FORMATION AND ITS ENHANCEMENT BY IFN

A Production of CTXs and of IFN in Response to the Same Inducing Agents

A considerable number of agents were found to induce the production of CTXs. As demonstrated in Fig. 2, effective production of CTXs can be induced in human peripheral blood mononuclear cells (PBMC) by agents as different from each other as phytohaemagglutinin (PHA) and Sendai virus. These two agents induce the proteins by affecting different kinds of leukocytes. Sendai virus induces the production of CTXs in MP, while PHA induces CTXs by stimulating non-adherent mononuclear cells (apparently T-cells) (Table II). Other T cell mitogens—such as foreign antigens—also induce CTXs in T cells (Ruddle *et al.*, 1985). In addition, bacterial lipopolysaccharides induce CTXs in MP (Ruff and Glifford, 1981a); the diterpene tumour promotor 4-β-phorbol-12-myristate-13-acetate (PMA) induces CTXs in MP as well as in certain lymphoid B and T cell lines (Gifford *et al.*, 1984; Williamson *et al.*, 1983; Adolf, 1984) and tumour cells can induce CTXs both in MP and in NK cells (Männel, 1981; Wright and Bonavida, 1982). The main CTX found to be produced by MP is TNF, while lymphocytes were reported to produce primarily LT (Chroboczek-Kelker *et al.*, 1985).

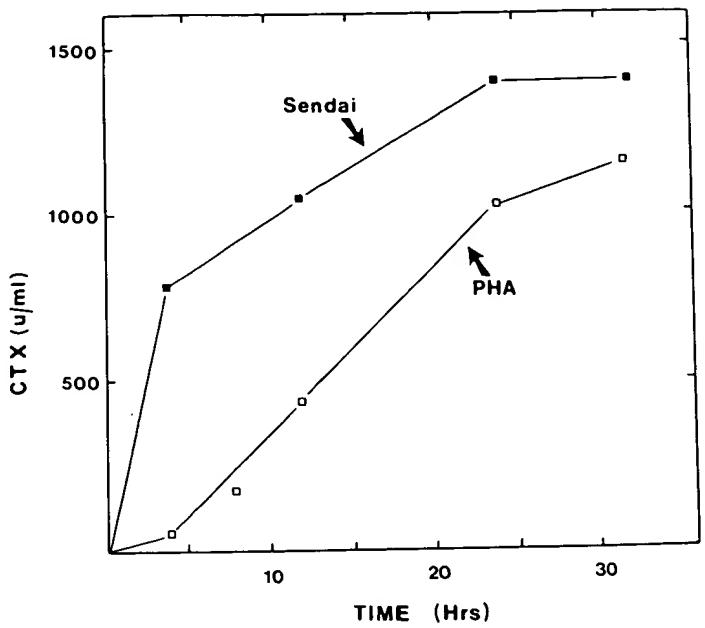


Fig. 2 Induction of CTXs in human PBMC (10^7 cells/ml) with Sendai virus (500 HA/ml) and with PHA (5 μg/ml) (Aderka *et al.*, 1986a).

TABLE II Differing Responsiveness of Mononuclear Phagocytes and of the Nonadherent Fraction of PBMC to Different Inducers of CTXs

Inducer	MP	Nonadherent PBMC (Primarily T-lymphocytes)
	CTX Yields (U/ml)	
None	10	< 5
PHA (5 μg/ml)	15	95
Con-A (20 μg/ml)	15	35
LPS (10 μg/ml)	30	< 5
PMA (5 ng/ml)	330	10
Sendai virus (500 HA/ml)	3000	15
LPS + PMA	2600	10

CTX induction (for 24 hours) by various agents, in fractionated human peripheral-blood mononuclear cells (PBMC) (Aderka *et al.*, 1986a).

Agents that stimulate T lymphocytes may, however, be found to induce significant production of TNF as well in cultures of PBMC, apparently through indirect stimulation of MP by the stimulated T cells (Aderka *et al.*, 1986a; Nedwin *et al.*, 1985). The identity of the CTXs comprising NKCF preparations is not yet clear. There have been preliminary reports suggesting that these preparations may contain TNF as well as CTXs that differ from TNF (Svedersky *et al.*, 1985a; Ortaldo *et al.*, 1985).

Those agents that induce production of CTXs in the various leukocytes also induce IFNs in the same cells (Table I): T cell mitogens induce in T cells the production of IFN-γ together with LT. Agents that stimulate MP to produce TNF also induce in these cells IFN-α and IFN-β, while in NK cells tumour cells induce the production of IFN-α as well as NKCF. Indeed, significant amounts of IFN would be detected in preparations of CTXs (see Matthews 1979; Ware and Granger, 1979).

B Enhancement of CTX Production by IFN

To find out if the IFNs produced with CTXs have a regulatory role in CTX induction, we have compared the efficiency of CTX production by PBMC pretreated with IFN-β to CTX production by untreated cells. No CTXs were induced as a result of treatment by IFN. However, when challenged by PHA, the IFN-treated cells produced CTXs at a significantly higher rate than untreated cells (Wallach and Hahn, 1983) (Fig. 4A). Furthermore, it could be shown, using antibodies to IFN, that production of CTXs by PHA-stimulated leukocytes that had not been pretreated by IFN was augmented, during prolonged incubation, by the IFN-γ produced *in situ* (Wallach *et al.*, 1983).

Enhancement of CTX production by IFN has turned out to be the rule for a variety of processes in which CTXs are formed, including the induction of CTXs in PBMC by antibody-coated cells IL-2, Concanavalin A (Con A), or staphylococcal enterotoxin A where, with the use of specific antibodies to TNF and LT it could be shown that production of both these CTXs were enhanced (Wallach *et al.*, 1983; Svedersky *et al.*, 1985b; Nedwin *et al.*, 1985). Production of TNF by mononuclear phagocytes in response to LPS and of NKCF by NK cells in response to tumour target cells was also found to be enhanced by IFN (Kildahl-Anderson *et al.*, 1985; Farram and Targan, 1983; Wright and Bonavida, 1983a; Steinhauer *et al.*, 1985). Furthermore, injection of IFN-γ into mice potentiated the production of TNF in subsequent injection of LPS (G. E. Gifford, and M. L. Lohmann-Matthes, personal communication). Augmentation *in vivo* of TNF production by IFN in man could be observed in patients with hairy-cell leukemia. The PBMC of these patients produce TNF poorly in response to stimulation *in vitro* by PHA, PMA or

Sendai virus. However, following a [illegible] tion of TNF in response to PHA increases. This augmentation appears to reflect and in responsiveness of the cells to i 1986b).

C Mechanisms Involved

There is inherent complexity in the pro regulation. Within the CTX-producing differing subcellular sites take part in the initial interaction of the cell with a C of the CTXs. Furthermore, indirectly, CTX-producing cell may also affect formation may be induced by cell-assoc gens on the surface of cells), whose expre functioning within such CTX-inducing production of CTXs. In addition, as CTXs may be affected by regulatory ce modulated by regulatory agents. Thes regulation by IFN of the processes in

Direct information on mechanisms i scarce. However, based on indirect info and components likely to take part in order to point out effects of IFN to modulated. These putative sites for re as well as the mode of regulation by IF diagrammatically in Fig. 3.

1 *Regulation of CTX formation a*

Since CTX-inducing agents are primar of CTXs occurs within the cell, there sensing mechanisms for these agents Such sensors are the T cell antigen re the induction of CTXs in T cells by to take part in induction of CTXs as (ADCC); membrane structures with CTXs in MP; and others. We know intracellular mediating mechanisms f tion. Cyclic AMP, a mediator in man involved, at least not as a positive

Sendai virus. However, following a month of treatment by IFN-α, production of TNF in response to PHA increases, reaching close to normal values. This augmentation appears to reflect both increase in counts of MP and in responsiveness of the cells to induction of TNF (Aderka *et al.*, 1986b).

C Mechanisms Involved

There is inherent complexity in the processes of CTX induction and in their regulation. Within the CTX-producing cell, components and mechanisms at differing subcellular sites take part in the sequence of events that lead from the initial interaction of the cell with a CTX inducer to the eventual secretion of the CTXs. Furthermore, indirectly, mechanisms functioning outside the CTX-producing cell may also affect the formation of CTXs. Since CTX formation may be induced by cell-associated components (e.g. foreign antigens on the surface of cells), whose expression can be modulated, mechanisms functioning within such CTX-inducing cells may have a regulatory role in the production of CTXs. In addition, as discussed below, the production of CTXs may be affected by regulatory cytokines, whose own formation can be modulated by regulatory agents. There are thus multiple potential sites for regulation by IFN of the processes involved in CTX induction.

Direct information on mechanisms that participate in CTX production is scarce. However, based on indirect information I list below cellular activities and components likely to take part in the processes of CTX induction, in order to point out effects of IFN through which these processes can be modulated. These putative sites for regulation of CTX production by IFN, as well as the mode of regulation by IFN of CTX effects on cells, are depicted diagrammatically in Fig. 3.

1. Regulation of CTX formation at the level of CTX-producing cells

Since CTX-inducing agents are primarily extracellular, while the production of CTXs occurs within the cell, there are likely to be cell surface receptors or sensing mechanisms for these agents that initiate the production of CTXs. Such sensors are the T cell antigen receptors, which are probably involved in the induction of CTXs in T cells by antigens; the Fc receptor, which is likely to take part in induction of CTXs as part of antibody-dependent cytotoxicity (ADCC); membrane structures with which LPS interacts when inducing CTXs in MP; and others. We know very little about the nature of the intracellular mediating mechanisms that transduce the signal for CTX induction. Cyclic AMP, a mediator in many hormonal processes, is unlikely to be involved, at least not as a positive signal, since agents that increase cyclic

AMP level in leukocytes have been found (Wallach *et al.*, 1983).

One may speculate, on the basis of effects the production of CTXs by lymphocytes the diterpene ester PMA to induce CTXs *et al.*, 1983; Williamson *et al.*, 1983; Gifford calcium fluxes or protein phosphorylation (known to be activated by PMA) play production of CTXs by these cells.

So far, the only intracellular event use of a molecular probe is the transcription and LT could be detected in CTX-pro cDNA probe (Gray *et al.*, 1984 and S injection of the RNA into *Xenopus* oocy biologically active TNF molecules (Wall either of these techniques, significant induction could be detected, indicating level of synthesis, processing or degradation and Svedersky *et al.*, 1985b).

Little information is available as to in CTX induction. In the formation of involving the removal of a long signal of these molecules from the cell. The s have not been identified as yet. Inhibi protein synthesis and cytoskeletal mod lectin-stimulated lymphocytes, and a raised against partially purified preparat as suggesting that CTXs can accumulate and on the cell surface (see Hiserodt Evidence for intracellular accumulation been presented based on detection of these cells (Wright and Bonavida, 19 cellular stores is also indicated by the the large granules of NK cells to be (Ortaldo *et al.*, 1985). Furthermore purified MP-produced CTXs were sim teins in mononuclear phagocytes (Kato monospecific antibodies to CTXs sho detailed information on the subcellular producing cells and on the mechanism In an attempt to clarify the IFN-m the enhancement of CTX formation

Fig. 3 Putative points of regulation by IFN (denoted by asterisks) in processes of CTX formation and in the response of target cells to the effect of CTXs.

AMP level in leukocytes have been found to suppress the formation of CTXs (Wallach *et al.*, 1983).

One may speculate, on the basis of effects of calcium modulating agents on the production of CTXs by lymphocytes, and on the basis of the ability of the diterpene ester PMA to induce CTXs in MP and in lymphocytes (Wallach *et al.*, 1983; Williamson *et al.*, 1983; Gifford *et al.*, 1984 and Adolf, 1984), that calcium fluxes or protein phosphorylation initiated by protein kinase C (known to be activated by PMA) play a role as mediating signals in the production of CTXs by these cells.

So far, the only intracellular event in CTX formation monitored with the use of a molecular probe is the transcription of CTX genes. mRNAs for TNF and LT could be detected in CTX-producing cells by hybridization to a cDNA probe (Gray *et al.*, 1984 and Svederksy *et al.*, 1985b) as well as by injection of the RNA into *Xenopus* oocytes, where it could be translated to biologically active TNF molecules (Wallach *et al.*, 1984, and Fig. 1C). With either of these techniques, significant increases in CTX mRNA upon CTX induction could be detected, indicating regulation of CTX formation on the level of synthesis, processing or degradation of mRNA (Wallach *et al.*, 1984 and Svedersky *et al.*, 1985b).

Little information is available as to the nature of post-translational events in CTX induction. In the formation of TNF, post-translational processing, involving the removal of a long signal peptide, has to occur prior to release of these molecules from the cell. The site and mechanism of this processing have not been identified as yet. Indirect evidence based on the effects of protein synthesis and cytoskeletal inhibitors on the release of CTXs by lectin-stimulated lymphocytes, and localization studies using antibodies raised against partially purified preparations of these CTXs, were interpreted as suggesting that CTXs can accumulate in lymphocytes both intracellularly and on the cell surface (see Hiserodt *et al.*, 1977, 1979).

Evidence for intracellular accumulation of NKCF in NK cells has also been presented based on detection of NKCF-like activity in homogenates of these cells (Wright and Bonavida, 1983a). The presence of NKCF in intracellular stores is also indicated by the ability of antibodies to preparations of the large granules of NK cells to block the cytotoxic activity of NKCF (Ortaldo *et al.*, 1985). Furthermore, antibodies raised against partially purified MP-produced CTXs were shown to interact with intracellular proteins in mononuclear phagocytes (Kildahl-Andersen *et al.*, 1985). The use of monospecific antibodies to CTXs should now generate more reliable and detailed information on the subcellular localization of CTXs within CTX-producing cells and on the mechanisms of their release.

In an attempt to clarify the IFN-regulated mechanisms that take part in the enhancement of CTX formation by lectin-stimulated PBMC pretreated

Fig. 3 Putative points of regulation by IFN (denoted by asterisks) in processing of CTXs.

with IFN, we first questioned whether the IFN-induced changes in this case occur in the CTX-producing cells themselves or are the result of a secondary effect, due to altered production of regulatory cytokines secreted by some leukocytes within the heterogeneous population of the PBMC. Preliminary data (Fig. 4) suggest that lectin-stimulated PBMC secrete cytokines, distinct from IFN (or CTXs), that enhance CTX formation as effectively as IFN. When applied on PBMC, IFN-free preparations of these cytokines did not induce production of CTXs (except at significantly higher concentrations than used in our experiments). However, on subsequent stimulation by PHA, PBMC treated at the lower concentrations of cytokines responded much more rapidly than non-treated cells in release of CTXs. In fact, this occurred just as rapidly as CTX release from IFN-pretreated cells (Fig. 4A).

The amounts of cytokines responsible for this effect could be estimated indirectly by determining how effectively the cytokine preparations enhanced CTX formation (Fig. 4B). Thus, it was shown that IFN-pretreated leukocytes produce more of these cytokines than cells that were not treated by IFN (Fig. 4C). Although the cytokines responsible for this effect have not yet been identified, the findings in this experimental system indicate that IFN-induced enhancement of CTX formation, at least in part, indirectly reflects enhanced formation of cytokines which themselves have a stimulatory effect on CTX formation. This complex mode of regulation by IFN may result in enhanced, cascade-like, stimulatory effect.

CTX formation and function may also be modulated by suppressive factors. The delayed type hypersensitivity (DTH) response, which probably involves CTX formation (see below), can be effectively blocked by suppressor cells. The decreased activity of suppressor cells, observed following treatment by IFN (Knop *et al.*, 1982) may further contribute to an increase in formation of CTXs.

Regulatory cytokines may function also as direct inducers of CTXs. Interleukin-2 was reported to induce effective production of both TNF and LT in cultures of PBMC (Svedersky *et al.*, 1985b; Nedwin *et al.*, 1985). IFN may increase the formation of interleukin-2 by enhancing the production of interleukin-1 and thus indirectly potentiate CTX production.

Modulation of production of regulatory cytokines is likely to contribute to a significant extent to the effect of IFN on formation of CTXs in cultures of mixed populations of leukocytes, where the differing kinds of mononuclear leukocytes indeed have marked regulatory effects on each other through the action of cytokines they secrete. To find out whether direct effects of IFN on CTX-producing cells may also contribute to the enhancement of CTX formation, we have sought to examine this regulation in a simpler experimental system—a homogeneous population of CTX-producing cells of cultured lines. Preliminary results from a study on the effect of IFN on the production

TABLE III Increase in Production of CTXs by U93 IFN, TNF or a Crude Leukocyte-produce

Preincubation	no inducing
–	< 5
PMA	10
rIFN-γ	< 5
PMA + IFN-γ	35
PMA + TNF	10
PMA + TNF + IFN-γ	40
PMA + crude leukocyte cytokines	N.T

U937 cells (10^6 cells/ml) in samples of 2 ml were incub 24 hours with the agents indicated in the first column, ther further for 24 hours either alone or with LPS or with Send were then determined by measuring cytotoxic effect on applied at 5 ng/ml, rIFN-γ at 1000 U/ml, TNF (native; hon at 100 U/ml, crude leukocyte cytokines (see below) at 0 virus at 500 HA/ml. Leukocyte cytokines were induced ir PMA (5 ng/ml) and Con-A (20 μg/ml), then concentrated and treated at pH 2.0 as described in the legend to Fig. detected in this kind of preparation (unpublished obser *N.T.—not tested.

of CTXs by the human histiocytic lymphon Table III. Although unable to produce CT ditions, the U937 cells do produce CTXs (pr differentiate with PMA, and produce CTXs further treated either with LPS or with Send IFN-γ enhances formation of the CTXs by tl this case an indirect effect of IFN through ir the U937 cells both produce and respond tc more likely that IFN enhances the productic inducing changes in cellular mechanisms dir CTX formation. Effects of regulatory cyto production may also be studied in the U937 production of CTXs (actually its own proc potentiation effect is additive to the effect c leukocyte-produced cytokines, free of both strong potentiation effects on the produ (Table III).

Among the various components and mecha cell whose alteration by IFN may result i CTXs, one may first point out two spec

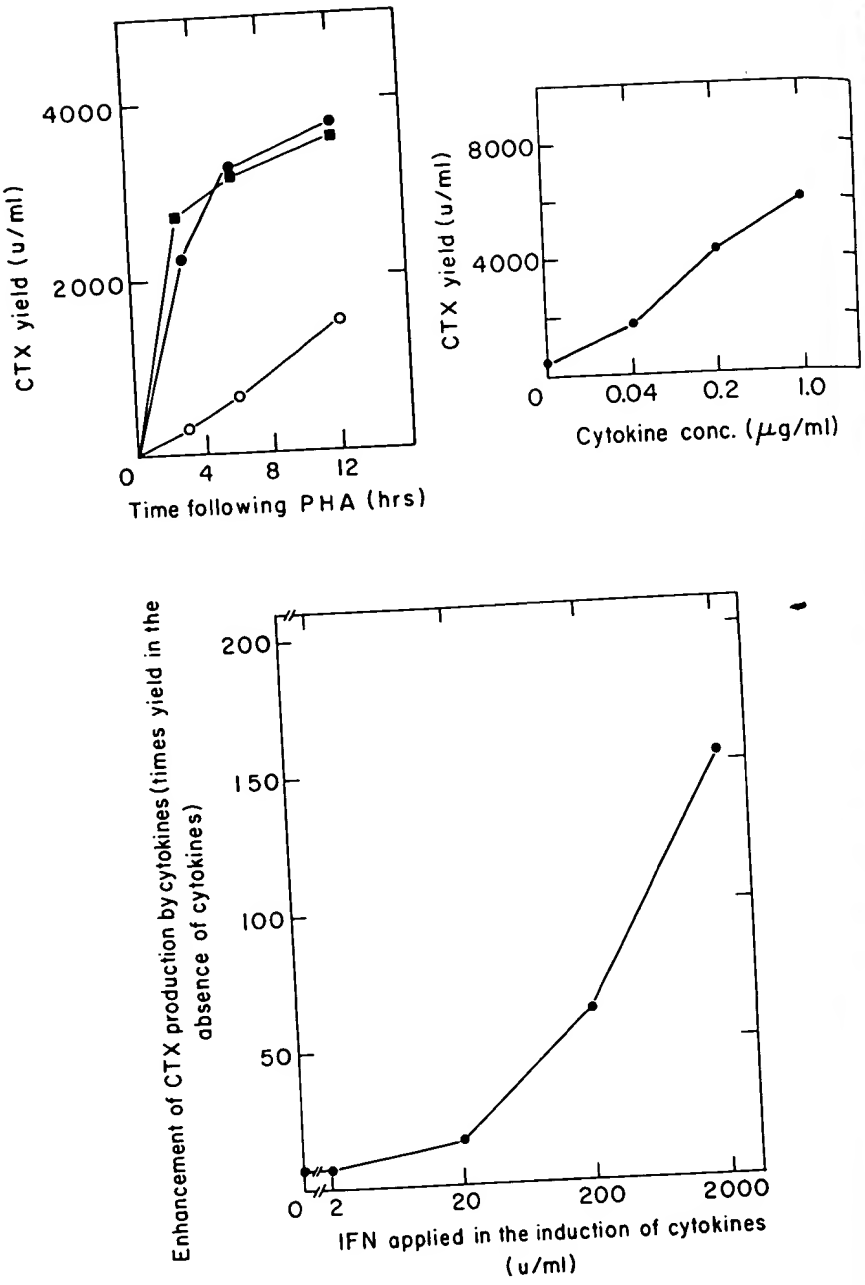
TABLE III Increase in Production of CTXs by U937 Cells Following Treatment by IFN, TNF or a Crude Leukocyte-produced Cytokine Preparation.

Preincubation	CTX yield (U/ml)		
	no inducing agents	LPS	Sendai virus
–	< 5	< 5	< 5
PMA	10	180	125
rIFN-γ	< 5	< 5	< 5
PMA + IFN-γ	35	500	625
PMA + TNF	10	440	N.T.*
PMA + TNF + IFN-γ	40	1030	N.T.
PMA + crude leukocyte cytokines	N.T.	3130	N.T.

U937 cells (10^6 cells/ml) in samples of 2 ml were incubated in 18 mm tissue culture wells for 24 hours with the agents indicated in the first column, then rinsed, brought to 1 ml and incubated further for 24 hours either alone or with LPS or with Sendai virus. Yields of CTXs in the cultures were then determined by measuring cytotoxic effect on CHI sensitized L132 cells. PMA was applied at 5 ng/ml, rIFN-γ at 1000 U/ml, TNF (native; homogeneously purified on the CT-1 mAb) at 100 U/ml, crude leukocyte cytokines (see below) at 0.2 μg/ml, LPS at 10 μg/ml and Sendai virus at 500 HA/ml. Leukocyte cytokines were induced in PBMC by 36 hours' stimulation with PMA (5 ng/ml) and Con-A (20 μg/ml), then concentrated by adsorption to controlled- pore glass and treated at pH 2.0 as described in the legend to Fig. 4. No IFN or CTX activity could be detected in this kind of preparation (unpublished observations).
*N.T.—not tested.

of CTXs by the human histiocytic lymphoma U937 cells are presented in Table III. Although unable to produce CTXs under normal growth conditions, the U937 cells do produce CTXs (primarily TNF) when induced to differentiate with PMA, and produce CTXs to an even greater extent when further treated either with LPS or with Sendai virus. As shown in Table III, IFN-γ enhances formation of the CTXs by the U937 cells. Although even in this case an indirect effect of IFN through induction of "autokines", which the U937 cells both produce and respond to, cannot be ruled out, it seems more likely that IFN enhances the production of CTXs in the U937 cells by inducing changes in cellular mechanisms directly involved in the process of CTX formation. Effects of regulatory cytokines other than IFN on CTX production may also be studied in the U937 cells. TNF itself potentiates the production of CTXs (actually its own production) in these cells, and that potentiation effect is additive to the effect of IFN-γ. Crude preparations of leukocyte-produced cytokines, free of both IFN and TNF, also have very strong potentiation effects on the production of CTXs by those cells (Table III).

Among the various components and mechanisms within the CTX-producing cell whose alteration by IFN may result in augmented production of the CTXs, one may first point out two specific receptors involved in these



processes: the Fc receptor, whose [illegible] augmented production of CTXs [illegible] tions, and the IL-2 receptor, which [illegible] is likely to be involved in the [illegible] well as by IL-2 itself. Recent findings consistent with the idea that transcription enhanced by IFN. The level of mRNA by IL-2 to produce CTXs was estimated for LT. In non-stimulated PBMC [illegible] below the level of detection; however, [illegible] accumulation of the mRNA could be [illegible] of IFN on the production of LT [illegible] not increase on treatment by IFN γ [illegible] significantly the increase in mRNA [illegible] with IL-2.

Fig. 4 IFN-induced increase in the production [illegible] formation. (A) Kinetics of CTX production [illegible] following pretreatment in IFN-α (1000 U/ml) [illegible] cytokine preparation (0.2 μg/ml) (■), compared [illegible] IFN nor by cytokines (○). (B) Titration of the [illegible] production of CTXs by PBMC in response [illegible] increase in production of the cytokines which [illegible] PBMC isolated from freshly donated blood [illegible] MEM-alpha medium at a concentration of [illegible] the cytokines and then rinsed and treated with [illegible] medium was determined with the use of [illegible] were produced by treating PBMC for 12 hours [illegible] concentrations in C1 then rinsing the cells [illegible] 20 μg/ml) for 24 hours (6 hours in C1). In A and B [illegible] by adsorption to controlled pore glass, eluted with [illegible] concentrated by ultrafiltration and incubated for [illegible] in the adsorption to controlled pore glass. A control [illegible] CTX activity of the cytokine preparation. [illegible] cytokines enhanced CTX formation. Depletion [illegible] tions on an immunoadsorbent constructed from [illegible] not decrease the effectiveness by which these cells [illegible] Inclusion of 50 mM α-methyl-D-mannopyranoside [illegible] function of any residual Con A that may [illegible] concentration in the crude cytokine preparation [illegible] the preparations of cytokines produced by [illegible] and then stimulated for 6 hours with Con A [illegible] just brought to pH 2.9 and 12 hours later [illegible] at a dilution of 1:5 on PBMC in the presence [illegible] were incubated with the lymphokines for 12 hours [illegible] PHA-P (5 μg/ml). Yields of CTXs were determined [illegible] ment by cytokines were 16 U/ml in B and 12 [illegible] was used in all experiments unpublished [illegible]

processes: the Fc receptor, whose increase by IFN may contribute to the augmented production of CTXs in antibody-dependent cytotoxic reactions, and the IL-2 receptor, which is also increased by IFN and which is likely to be involved in the induction of CTXs by T cell mitogens as well as by IL-2 itself. Recent findings by Sverdersky *et al.* (1985b) are consistent with the idea that transcription of mRNAs for CTXs is also enhanced by IFN. The level of mRNA for this CTX, in PBMC stimulated by IL-2 to produce CTXs, was estimated with the use of a cDNA probe for LT. In non-stimulated PBMC the concentration of mRNA for LT was below the level of detection; however, following treatment by IL-2, significant accumulation of the mRNA could be observed. In correlation with the effect of IFN on the production of LT itself, the mRNA for this protein did not increase on treatment by IFN-γ alone; but IFN treatment did enhance significantly the increase in mRNA observed on subsequent treatment with IL-2.

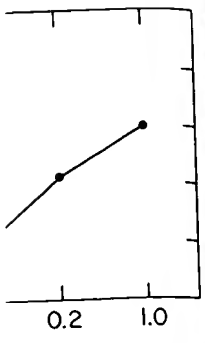


Fig. 4 IFN-induced increases in the production of CTXs and of cytokines which enhance CTX formation. (A) Kinetics of CTX production by PBMC in response to stimulation by PHA following pretreatment by IFN-α (1000 U/ml) (●) or by IFN-free leukocyte-produced crude cytokine preparation (0.2 μg/ml) (■), compared to CTX production in PBMC not pretreated by IFN nor by cytokines (○). (B) Titration of the stimulatory effect of the cytokine preparation on production of CTXs by PBMC in response to 6 hours' stimulation with PHA. (C) IFN-induced increase in production of the cytokines which enhance CTX formation.

PBMC isolated from freshly donated blood on a Ficoll-Hypaque cushion, were suspended in MEM-alpha medium at a concentration of 10^7 cells/ml, incubated for 12 hours with IFN or with the cytokines and then rinsed and treated with PHA-P (5 μg/ml). Cytotoxic activity in the culture medium was determined with the use of CHI-sensitized SV80 cells. The cytokine preparations were produced by treating PBMC for 12 hours with IFN-α (2000 U/ml in A, B and at varying concentrations in C) then rinsing the cells 3 times and applying Concanavalin A (Con-A 20 μg/ml) for 24 hours (6 hours in C). In A and B these cytokine preparations were concentrated by adsorption to controlled pore glass, eluted with 0.5 M tetramethylammonium chloride, further concentrated by ultrafiltration and incubated for 12 hours at pH 2.0. The ConA was eliminated in the adsorption to controlled pore glass. Acidification resulted in complete loss of IFN and of CTX activity of the cytokine preparation with no decrease in the effectiveness by which the cytokines enhanced CTX formation. Depletion of IFN-γ by applying these cytokine preparations on an immunoadsorbent constructed from a monoclonal antibody against that IFN did not decrease the effectiveness by which those cytokines augmented production of CTXs either. Inclusion of 50 mM α-methyl-D-mannopyranoside during CTX induction, to interfere with the function of any residual Con A, did not decrease the enhancement of CTX formation. Protein concentration in the crude cytokine preparation was about 1 μg/ml of leukocyte culture. In C, the preparations of cytokines (produced by cells pretreated with various concentrations of IFN-α and then stimulated for 6 hours with Con A) were not concentrated on controlled pore glass but just brought to pH 2.0 and 12 hours later returned to pH 7.0. The preparations were then applied at a dilution of 1 : 5 on PBMC in the presence of α-methyl-D-mannopyranoside (50 mM). The cells were incubated with the lymphokines for 12 hours and, after rinsing, for 6 further hours with PHA-P (5 μg/ml), yields of CTXs were determined. Yields of CTXs in the absence of pretreatment by cytokines were 68 U/ml in B and 11 U/ml in C. Electrophoretically pure native IFN-α was used in all experiments (unpublished observations).

2 Regulation at the level of CTX inducing cells

Additional potential sites for regulation by IFN can be discerned in those processes for CTX production in which the inducing agents are cells—cells which present foreign antigens on their surface and which induce CTXs in T lymphocytes, and tumour cells that induce CTXs in NK cells and in mononuclear phagocytes. The role of IFN in the production of CTXs by antigen-stimulated T cells has not, to my knowledge, yet been examined. In theory though, we may expect, that IFN will effectively modulate these processes of CTX production by inducing two kinds of changes: increase in major histocompatibility (MHC) proteins, and the antiviral effect, neither when induced in the CTX-producing T cells but in the cells that present the stimulating antigens to the T-cells.

As in other processes of antigen-induced T cell activation, in the induction of CTXs in T cells, antigens have to be presented in association with the MHC proteins. This refers not only to cell-associated foreign antigens, but also to soluble antigens, since these are recognized by T cells only after being processed (primarily by MP) and exposed on the surface of the processing cell in association with MHC proteins.

Of the two major classes of the MHC proteins (Class I proteins HLA-A, -B, -C in man and H2-K and -D in mouse, and Class II proteins including HLA-DR in man and Ia in mouse), it seems that the class II proteins primarily take part in CTX induction. After fractionation of T cells, most cells capable of producing CTXs in response to antigens were Lyt-1^+ cells, known to recognize antigens in the context of the class II MHC proteins. Little CTX induction could be detected in the Lyt-2^+ cells, which recognize antigens associated with the class I MHC proteins (Eardley *et al.*, 1980; Tite *et al.*, 1985). IFNs, most notably, IFN-γ, enhance synthesis of the MHC proteins, particularly the class II MHC proteins (Lindahl *et al.*, 1974; Wallach, *et al.*, 1982; Basham and Merigan, 1983).

In a variety of cells, synthesis of the class II MHC proteins may, in fact, be observed only upon treatment by IFN-γ. IFN may in this way greatly promote antigen-induced CTX production. However, IFN may suppress induction of CTXs by antigens when these are virus-derived. This may not be an exceptional situation, since infecting viruses are probably the most common physiological source of cell-associated foreign antigens. Many viruses, however, do not respond to the antiviral effect; cell surface expression of viral antigen may even be found to be increased by IFN (Chang *et al.*, 1977).

On the whole, the combined effects of IFN on the MHC antigens and on the expression of viral antigens on the cell surface may be expected to result in decreased CTX induction by virus-infected cells whenever IFN suppresses

replication of the virus, and in enhanc
that fail to respond to the antivira
There is evidence that in the induc
a regulatory role at the level of the
which can induce the production of
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suppress NK cytotoxicity by induci
(Trinchieri and Santoli, 1978; Wallac
on the molecular nature of these IFN
on the nature of the target structure

IV CTX EFFECTS AND THE

A CTX Effects

1 Cytotoxic and cytostatic effects

In most studies on the cytotoxic
been L929 cells, which are particular
erization of the effect of CTXs o
wide spectrum of cells that are pote
most of these cells the cytotoxic effe
for several days. A rapid cytotoxic effe
application, would not be observe
in some way. Such sensitization ca
metabolic blockers such as actinomyc
0-dinitrophenyl or sodium azide (Will
al., 1973; Ruff and Gifford, 1981b;
tions). It can also be induced by x-
treated cells with certain viruses (Eif
figs 5 and 6).

Cells of several lines, including some
toxicity of CTXs, can be found to resp
The relation of this cytostatic effect o
clear. Possibly growth arrest by these
collection of cellular changes that even
exist in some cells, growth arrest by C
growth-related functions, as indicated
tion of cells in specific stages of the cel
respond to TNF in an arrest at G

replication of the virus, and in enhanced induction of CTXs by infected cells that fail to respond to the antiviral effect of IFN.

There is evidence that in the induction of CTXs in NK cells, too, IFN has a regulatory role at the level of the CTX-inducing cells. Mouse Yac cells, which can induce the production of NKCF, become less effective inducers when treated by IFN (Wright and Bonavida, 1983b). That observation is consistent with prior studies showing that IFNs, most notably IFN-γ, can suppress NK cytotoxicity by inducing resistance to killing in the target cells (Trinchieri and Santoli, 1978; Wallach, 1983). There is as yet no information on the molecular nature of these IFN-induced changes in NK-target cells or on the nature of the target structures for NK-cells in general.

IV CTX EFFECTS AND THEIR POTENTIATION BY IFN

A CTX Effects

1 Cytotoxic and cytostatic effects

In most studies on the cytotoxic effect of CTXs, the target cells have been L929 cells, which are particularly sensitive. From the limited characterization of the effect of CTXs on other cells, it appears that there is a wide spectrum of cells that are potentially sensitive to CTXs. However, in most of these cells the cytotoxic effect is rather slow, occurring in incubation for several days. A rapid cytotoxic effect, occurring within a few hours of CTX application, would not be observed unless the cells had been sensitized in some way. Such sensitization can be achieved by treating cells with metabolic blockers such as actinomycin D, cycloheximide (CHI), mitomycin-c, 2,4-dinitrophenyl or sodium azide (Williams and Granger, 1973; Rosenau *et al.*, 1973; Ruff and Gifford, 1981b; Wallach, 1984, unpublished observations). It can also be induced by x-irradiation or by infection of the CTX-treated cells with certain viruses (Eifel *et al.*, 1979; Aderka *et al.*, 1985; and Figs 5 and 6).

Cells of several lines, including some that are quite resistant to the cytotoxicity of CTXs, can be found to respond to CTXs by arrest of cell growth. The relation of this cytostatic effect of CTXs to the cytotoxic activity is not clear. Possibly growth arrest by these proteins is, in many cases, an early reflection of cellular changes that eventually result in cell death. However, at least in some cells, growth arrest by CTXs may reflect specific inhibition of growth-related functions, as indicated by the fact that it results in accumulation of cells in specific stages of the cell cycle. Thus L929 cells were reported to respond to TNF in an arrest at G_2 (Darzynkiewicz *et al.*, 1984), and B16

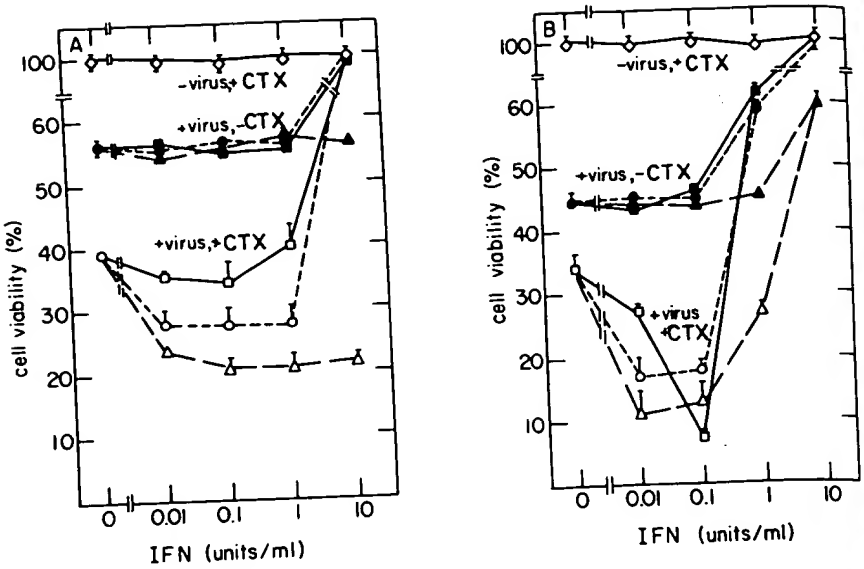


Fig. 5 IFN effects on the cytotoxicity of CTXs in SV80 cells (A) and in HeLa cells (B), IFN-α (○, ●), IFN-β (□, ■), and IFN-γ (△, ▲) were applied at the indicated concentrations prior to infection by VSV (solid symbols) or infection by VSV followed by application of CTX (6 U/ml, open symbols) or application of CTXs alone (6 U/ml, for the three types of IFN, ◇) (see note in Fig. 6 on the way the CTXs were induced). Note the enhancement of cell-killing at low concentrations of IFN as opposed to the protective effect of IFN at higher concentrations (Aderka *et al.*, 1985).

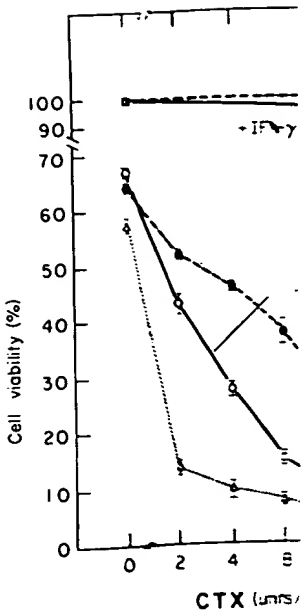


Fig. 6 Enhancement by IFN-γ of CTX cytote effect of CTXs at various concentrations in VS by treating these cells with IFN-γ (10 U/ml) (C shown in comparison to the resistance to the C treated with IFN-γ (□). The CTXs used in the e preparations of CTXs induced by PHA in PBM pore glass and fully depleted of IFN-γ by the use Selective cytotoxicity against VSV infected cells observed with use of pure TNF.

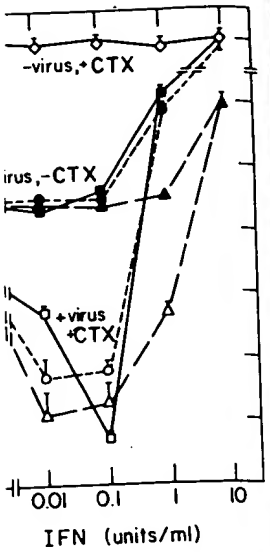
melanoma cells responded to purified LT, and more effectively to LT plus IFN-γ, in an arrest at the G_0/G_1 phase (Lee *et al.*, 1984). Indeed, there is preliminary evidence that transcription of the c-*myc* oncogene is in certain tumour cells, effectively turned off by TNF (Yarden, A., Wallach, D. and Kimchi, A. submitted).

2 *Protective and cell growth promoting effects*

Some of the molecular changes observed in cells treated by CTX are inconsistent with the cytotoxic and cytostatic functions of these proteins. Rather than interfering with viable functions of the cells, CTXs have been found to induce an increase in RNA, protein and lipid synthesis (Rosenau, 1980; Ostrove and Gifford, 1979). Furthermore, in cells resistant to the cytotoxic effect of CTXs, these proteins could even be found to stimulate cell multiplication (Sugarman, *et al.*, 1985; Fiers *et al.*, 1986; Vilček *et al.*, 1986). As discussed below, it appears that these "constructive" effects of CTXs in part reflect repair mechanisms that counteract the cytotoxic activity of these proteins, thus preventing non-selective cell destruction. It also seems possible that these CTX effects contribute to n to the increased cell growth necessar areas, where at an earlier stage CTX

3 *CTXs as pleiotropic mediators*

Apart from effects on cell growth and been found to exert a variety of other are immunoregulatory effects; prim immune cytolysis. LT was reported ADCC and phagocytic activities (Sha ADCC activity in the U937 hystioc 1985b); it also enhances the producti There is some evidence that TNF can T lymphocytes (cf. Fiers *et al.*,



) and in HeLa cells (B), IFN-α
icated concentrations prior to
y application of CTX (6 U/ml,
ree types of IFN, ◇) (see note
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IFN at higher concentrations

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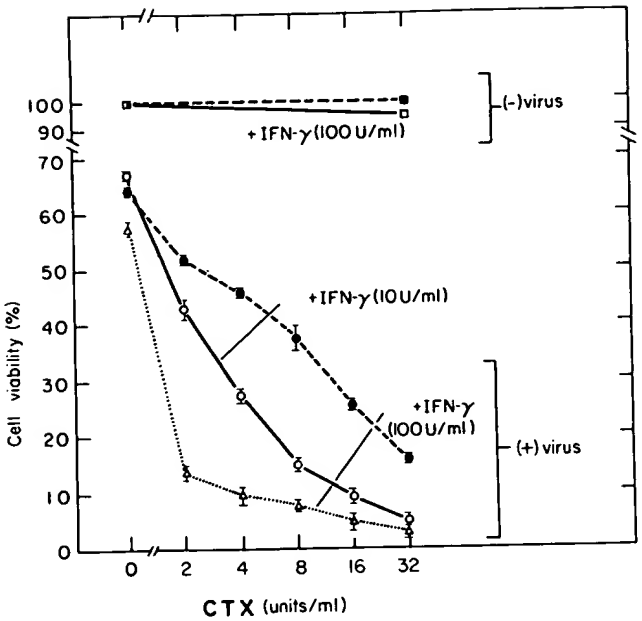

Fig. 6 Enhancement by IFN-γ of CTX cytotoxicity in VSV-infected SV80 cells. The cytotoxic effect of CTXs at various concentrations in VSV-infected cells (●) and its further enhancement by treating these cells with IFN-γ (10 U/ml) (○) or 100 U/ml (△)) 16 hours prior to infection is shown in comparison to the resistance to the CTXs observed in uninfected cells (■) even when treated with IFN-γ (□). The CTXs used in the experiments described in Figs 5 and 6 were crude preparations of CTXs induced by PHA in PBMC, partially purified by adsorption to controlled pore glass and fully depleted of IFN-γ by the use of a monoclonal antibody (Aderka *et al.*, 1985). Selective cytotoxicity against VSV infected cells and its augmentation by IFN could similarly be observed with use of pure TNF.

that these CTX effects contribute to repair on the multicellular level, namely to the increased cell growth necessary for healing the damage in necrotic areas, where at an earlier stage CTX effects have led to the death of cells.

3. *CTXs as pleiotropic mediators*

Apart from effects on cell growth and viability, TNF and LT have recently been found to exert a variety of other effects on cell function. Some of these are immunoregulatory effects; primarily effects on functions related to immune cytolysis. LT was reported to induce in granulocytes enhanced ADCC and phagocytic activities (Shalaby, *et al.*, 1985a) and TNF to increase ADCC activity in the U937 hystiocytic lymphoma cells (Shalaby, *et al.*, 1985b); it also enhances the production of CTXs by those cells (Table III). There is some evidence that TNF can promote the differentiation of cytotoxic T lymphocytes (cf. Fiers, *et al.*, 1986). Furthermore, it may enhance

cell-killing by cytotoxic T lymphocytes through effects on the target cells: TNF increases the synthesis and cell surface expression of Class I MHC antigens (Collins, *et al.*, 1986) and, according to a preliminary report, also of Class II antigens (Chang and Lee, 1985). Cells that have been exposed to TNF may, therefore, be more effectively recognized by the MHC-restricted cytotoxic T lymphocytes.

Quite a different role for TNF was revealed in the recent identification of TNF with the "cachectin"—a cytokine which suppresses in adipocytes the activity of lipoprotein lipase and also induces in these cells a decrease of mRNAs for some other proteins involved in the differentiated function of the adipocyte (Beutler, *et al.*, 1985a; Torti, *et al.*, 1985). Apparently that effect of TNF contributes to the defence against disease by assuring the availability of lipids for rapid use. However, when induced for prolonged duration the "cachectin" effect of TNF may result in an excessive and dangerous weight loss (cachexia).

That TNF can mediate some deleterious effects in diseases is also indicated by a recent study showing that injected antibodies to TNF reduce in mice the lethal effect of bacterial endotoxins. This indicates that TNF has a causative role in bacterially-induced septic shock (Beutler *et al.*, 1985b).

Since this review was submitted for publication (15 November) until to date (June 1986) many additional effects of TNF on cell functions have been revealed. Quite an unexpected finding, derived both from the studies on TNF and from studies on interleukin-1 (IL-1), is that these two cytokines, which bare no similarity of structure and apparently function through binding to distinct receptors, affect cell function in quite a similar manner. IL-1 can actually be regarded as a cytotoxin-certain tumour cells are killed by IL-1 as effectively as by LT (Onozaki, *et al.*, 1985), IL-1 also shares the ability of TNF to stimulate growth of fibroblasts (Schmidt, *et al.*, 1982). Some other examples of the similarity in function of TNF and IL-1 are: (1) Induction, by both, of the synthesis of collagenase and of prostaglandin E_2, in fibroblasts (Dayer, *et al.*, 1984, 1985); (2) The "cachectin"-lime activity (suppression of lipoprotein lipase in adipocytes) found to be mediated, not only by TNF but also by IL-1 (Beutler and Cerami, 1985); (3) Activation of osteoclasts by the two (Dewhirst, *et al.*, 1985; Bertolini, *et al.*, 1986), and (4) Induction in endothelial cells of increased adhesivity to granulocytes and of synthesis of a cell surface protein which probably is involved in that adherence (Gamble, *et al.*, 1985; Schleimer and Rutledge, 1986; Pober, *et al.*, 1986). Particularly interesting from the point of view of IFN research is the finding that both IL-1 and TNF induce in fibroblasts the synthesis of a specific IFN (IFN-β_2) (Content, *et al.*, 1985; Kohase, *et al.*, 1986). According to preliminary reports, TNF induces synthesis of an IFN also in PBMC (Wong and Goeddel, 1985).

B IFN-induced Enhancement

The cytotoxic and cytostatic
synergistically. An IFN-induced
could be observed with crude CTX,
LT (Williams and Bellanti, 19
Stone-Wolff *et al.*, 1984; Le
NKCF (Wright and Bonavida,
immunoregulatory effects of TNF
tiated by IFN (Shalaby *et al.*,

Yet, even though IFN
CTXs, it has so far not
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situation in which IFN
selectivity is presented in Figs 5
IFN-free CTX preparations
analysed, comparing cells
Under normal growth
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virus (cf. Fig. 6, curves ■ and ●),
infected cells but not to
uninfected cells (cf. Fig. 6,
IFN suppressed replication of
of the infected cells could
At higher IFN concentrations,
resulted in resurgence of the
in Fig. 5B, the enhancement of
to the protection from killing

C Mechanisms Involved

Radioactively tagged TNF and
cells; the number of binding
18,000 per cell, depending on
1985; Tsujimoto *et al.*, 1985,
cross-linking agents, specific
TNF bind, forming conjugates
Kull *et al.*, 1985).

These findings provide
cell surface receptors for CTXs.
those CTXs that function

B IFN-induced Enhancement of CTX Function

The cytotoxic and cytostatic effects of CTXs and those of IFNs are exerted synergistically. An IFN-induced increase in anticellular activities of CTXs could be observed with crude CTX preparations and with purified TNF and LT (Williams and Bellanti, 1983; Williamson *et al.*, 1983; Wallach *et al.*, 1983; Stone-Wolff *et al.*, 1984; Lee *et al.*, 1984) as well as with preparations of NKCF (Wright and Bonavida, 1983b; Steinhauer *et al.*, 1985). Furthermore, immunoregulatory effects of TNF and LT were also reported to be potentiated by IFN (Shalaby *et al.*, 1985a, b).

Yet, even though IFN appears generally to potentiate the function of CTXs, it has so far not been found to interfere with selectivity in this function by inducing responsiveness to CTXs in cells that in the absence of IFN are completely unresponsive to these proteins. An example of a situation in which IFN potentiates CTX activity without decreasing its selectivity is presented in Figs 5 and 6 in which the cytotoxic effect of IFN-free CTX preparations in SV-80 and HeLa cells is quantitatively analysed, comparing cells that were infected by VSV to uninfected cells. Under normal growth conditions the cells are quite resistant to killing by CTXs but they do respond to the cytotoxic effect following infection by the virus (cf. Fig. 6, curves ■ and ●). IFN is found to potentiate killing of the infected cells but not to impose vulnerability to this cytotoxic effect on the uninfected cells (cf. Fig. 6, curves □ and △). Since, at antiviral concentrations, IFN suppressed replication of the virus, an IFN-induced increase in killing of the infected cells could only be observed at sub-antiviral concentrations. At higher IFN concentrations, suppression of virus replication by IFN resulted in resurgence of the resistance to the effect of the CTXs (compare, in Fig. 5B, the enhancement of killing of HeLa cells by IFN-γ at 0.01 U/ml to the protection from killing when the IFN concentration was 10 U/ml).

C. Mechanisms Involved

Radioactively tagged TNF and LT bind to high-affinity sites on the surface of cells; the number of binding sites range from undetectable levels to as much as 18,000 per cell, depending on the cell line (Hass *et al.*, 1985; Kull *et al.*, 1985; Tsujimoto *et al.*, 1985, 1986; Israel *et al.*, 1986). Using bifunctional cross-linking agents, specific cell surface proteins could be observed to which TNF bind, forming conjugates with M_r of about 75 and 92 Kd (Fig. 7 and Kull *et al.*, 1985).

These findings provide direct evidence for the long-suspected existence of cell surface receptors for CTXs. A clear distinction can now be made between those CTXs that function through binding to specific receptors and other

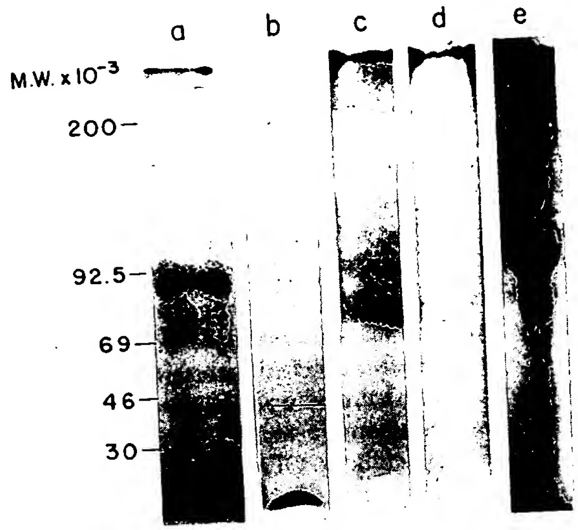

Fig. 7 Characterization of the receptors to human TNF on human U937 (a, b) and KG-1 (c–e) cells by their cross-linking to ^{125}I-TNF. a, b, e: cross-linking with disuccinimidyl suberate (in b-excess nonlabelled TNF was included during incubation with the labelled protein). c–d: cross-linking with the bifunctional agent dithiobis succinimidyl propionate which is cleavable with β-mercaptoethanol; analysis in the absence (c) and presence (d) of β-mercaptoethanol. Cells were incubated for 2 hours with ^{125}I-TNF (100 U/ml), which was radio-iodinated by the chloramine-T method. They were then washed, treated with the cross-linking agents and extracted with a buffer containing 2% Triton X-100. The extracts were analysed by SDS/7.5%-PAGE followed by autoradiography. The heavy radioactive band at the bottom of lane a is free TNF (Israel *et al.*, 1986).

cytotoxic proteins, also present in crude CTX preparations, that kill cells via enzymic activity (e.g. arginase or protease (Currie, 1978; Adams, 1980)). A third mode of cell-killing by leukocyte-produced proteins is exemplified in the function of the 'perphorins' (see Section II (B)) which, like the C_9 component of the complement, are believed to kill cells by forming channel-like structures in cell membranes, apparently with no prior interaction with any receptor protein.

The nature of the mechanisms activated by occupation of the TNF receptor, and which eventually lead to the functional changes induced by the CTXs, remains obscure. Similarly to cell surface receptors for many other agonists, the receptors for TNF are taken up into the cell after becoming occupied and the CTXs bound to these receptors are then degraded, apparently in lysosomes (Tsujimoto *et al.*, 1985). Clearly this uptake process has no role in the cytotoxic effect, as cytotoxicity can be effectively induced in the presence of sodium azide, which fully blocks the uptake (unpublished results). Neither does it appear that the cytotoxic effect is dependent on

synthesis of any protein or RNA in the target cell, since it occurs in the presence of azide, CHI or actinomycin D; these agents actually potentiate the cytotoxic effect of CTXs. The fact that the cytotoxic effect of CTXs is exerted in the presence of inhibitors of RNA, protein and ATP synthesis, also implies that the CTXs themselves do not possess such a general inhibitory capacity and that their cytotoxic function reflects another mode of action. Specific RNA and protein species do decrease in response to TNF, apparently owing to suppressed synthesis of these molecules (Torti *et al.*, 1985), but TNF and LT have also been found to induce an increase in the synthesis of cellular RNA, proteins and lipids (Rosenau 1980; Ostrove and Gifford 1979).

Thus, as in the function of various other regulatory cytokines, we are faced in the function of CTXs with transduction of information from the receptors for these proteins to the interior of the cell, in a so far unknown fashion, resulting in altered synthesis of specific species of RNA and protein molecules and yet those effects are apparently distinct from those participating in the cytotoxic effect of the CTXs. Actually, as mentioned above, it has been suggested that the enhancement of synthesis of RNA, protein and lipid by CTXs has a role in counteracting the cytotoxic effects which the CTXs themselves induce (Rosenau, 1980). The fact that metabolic inhibitors such as CHI and actinomycin D, which are likely to interfere with CTX-induced increases in anabolic activities, sensitize cells to the cytotoxic effect, is consistent with a protective role for these CTX-induced changes. In further support for the idea that CTXs activate mechanisms in cells that counteract their cytotoxicity, we have found that, within a short time after application of CTXs, cells become less responsive to sensitization by CHI (Fig. 8).

Such induction of resistance to CTX by the CTX itself could be observed with purified TNF (Hahn *et al.*, 1985) as well as with partially purified preparations of LT, free of TNF, the two kinds of preparations inducing mutual resistance as well as resistance to their own effect (unpublished observations). Auto-induction of resistance to TNF could not be related to depletion of receptors to the protein. As shown in Fig. 9, within a few hours of removal of TNF, which had been applied to the cell at saturating concentrations, free cell-surface receptors for the protein were replenished, yet the extent of resistance to TNF cytotoxicity induced by such pretreatment remained unchanged. It therefore seems likely that this resistance indeed reflects the activation by TNF of mechanisms that counteract its cytotoxic effects.

Recently we have noticed that, besides TNF and LT, several other cytokines, including insulin and interleukin-1 (IL-1) can induce in cells resistance to the cytotoxicity of the CTXs. Further examination of the protective effect of IL-1 revealed induction, by this cytokine, of a rapid and effective decrease in cell surface receptors to TNF. That decrease could not fully account for

the protective effect of IL-1 since following the removal of IL-1 TNF receptors were rapidly replenished yet the cells remained resistant to the cytotoxicity of TNF (Holtmann, Hahn and Wallach, submitted).

The potentiation of CTX effects by IFN can be related in part to an IFN-induced increase in the number of CTX receptors. As shown in Table IV, cells of several lines show an increase in binding of ^{125}I-TNF following treatment with IFN-γ. Some of these cells also display such an increase when treated by IFN-α. An IFN-induced increase in binding of TNF has also been recently observed by others (Tsujimoto *et al.*, 1986; Aggarwal *et al.*, 1985b and Ruggiero *et al.*, 1986). These studies indicate that this IFN effect reflects an increase in amounts of the receptor protein, apparently as a consequence of its increased synthesis. It is unlikely, though, that this moderate IFN-induced increase in receptor density fully accounts for the pronounced potentiation of CTX function by IFN (compare the increase in receptor level to cytotoxic effect in Fig. 10). Other IFN-induced changes are probably also involved. Perhaps the increased sensitivity of IFN-treated cells to the cytotoxic effect of CTXs reflects not only potentiation of the mechanisms mediating this effect but also suppression of cellular mechanisms that counteract it. Consistent with this idea, the induction of auto-resistance to

TABLE IV IFN-induced increase in receptors for TNF.

Cells	IFN (1000 U/ml)	CTX Binding (cpm/10^6 cells)	CTX Binding (relative increase)
HeLa	—	1080	1
	IFN-α	1800	1.67
	IFN-γ	2200	2.04
WISH	—	1050	1
	IFN-α	1000	0.95
	IFN-γ	1440	1.37
L-132	—	800	1
	IFN-α	1000	1.25
	IFN-γ	1040	1.3
FS11	—	440	1
	IFN-α	400	0.91
	IFN-γ	650	1.48
U937	—	920	1
	IFN-α	1300	1.41

Specific binding of radiolabelled TNF at a concentration of 2 U/ml (U937 cells) and 10 U/ml (other cells) was determined following 12 hours treatment by rIFN-α or rIFN-γ at 1000 U/ml (Israel *et al.*, 1985).

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ells) and 10 U/ml
N-γ at 1000 U/ml.

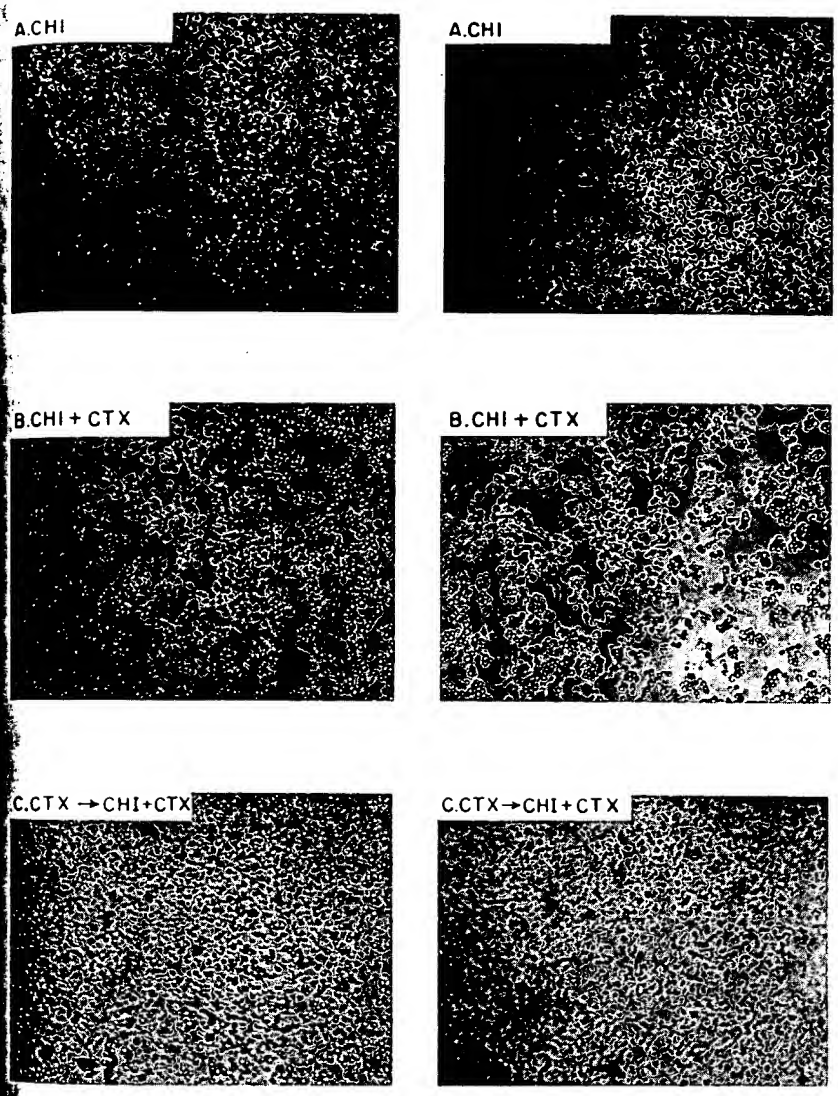

Fig. 8 Auto-induction of resistance to the cytotoxic effect of CTXs. The killing of HeLa and SV80 cells by a crude preparation of PHA-induced human CTXs (8 U/ml), incubated for 12 hours together with CHI (50 μg/ml) (B), compared with the normal morphology of the cells observed on incubation with CHI alone (A). The extent of killing is markedly reduced in cells which were pretreated for 3 hours with CTXs (80 U/ml) in the absence of CHI, rinsed and treated again with CTX (8 U/ml) this time in the presence of CHI (C) (Wallach, 1984).

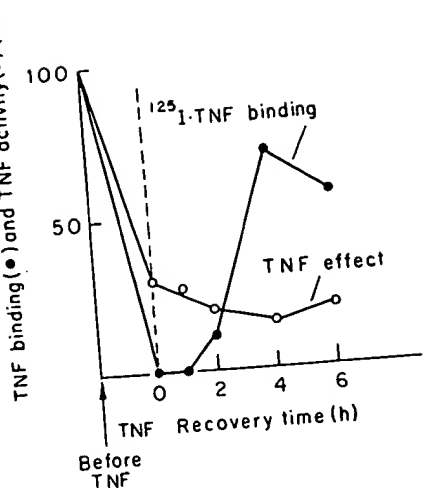

Fig. 9 Lack of correlation between TNF receptor concentration and decrease in vulnerability to its cytotoxic effect following pretreatment of L132 human cells with TNF. L132 human cells were incubated for 2 hours with TNF (260 U/ml), rinsed and further incubated in TNF-free medium. At various times the ability of the cells to bind TNF and to respond to its cytotoxic effect was measured; the former by binding of ^{125}I-labelled TNF (60 U/ml) and the latter by measuring the cytotoxicity of TNF (7 U/ml) in the presence of CHI. Results are presented at per cent of TNF bound specificially (960 c.p.m./10^5 cells) and of the cytotoxic titre of the test sample of TNF, observed before pretreatment by TNF (Israel *et al.*, 1986).

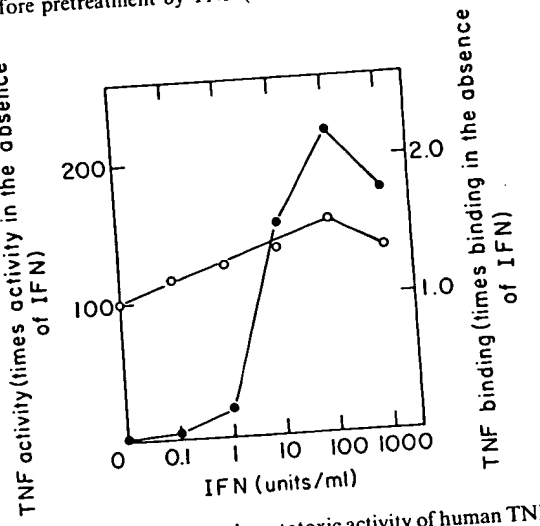

Fig. 10 Titration of the effect of IFN-γ on the cytotoxic activity of human TNF (solid symbols) and on the binding of the protein to its receptors (open symbols) in human HeLa cells. Cells were incubated for 12 hours with the indicated concentrations of IFN-γ. Their vulnerability to the cytotoxic effect of TNF was then determined by titrating; in the presence of CHI, the cytotoxic activity of a given preparation of TNF (titre of 530 U/ml in cells not treated by IFN). Binding of TNF was determined by incubating the cells with saturating levels of ^{125}I-TNF (7500 U/ml). In cells not treated by IFN, specific binding of 1000 c.p.m./10^5 cells was observed (Israel *et al.*, 1986).

TNF, described above, is found [illegible] (unpublished results).

V ON THE PHYSIOL[illegible]

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(2) Some of the leukocytes [illegible] of the cytotoxic T lymphocytes [illegible] those target cells to which they [illegible] in such "contact-dependent" [illegible] but remains to be confirmed. [illegible] take part, it is unlikely to be the [illegible] can be induced by both cellular [illegible] the way the CTXs function [illegible] should allow them to affect [illegible] and LT have a role as mediators [illegible] ducing cells.

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TNF, described above, is found to be greatly reduced in cells treated by IFN (unpublished results).

V ON THE PHYSIOLOGICAL ROLE OF THE CTXs

There is little information available on the formation and function *in vivo* of CTXs. The physiological role of these proteins can, therefore, only be a matter of speculation, on the basis of our present fragmentary knowledge of the way these proteins are formed and function *in vitro*. The following implications of that knowledge seem to me particularly worthy of emphasis.

(1) It seems reasonable to assume that the CTXs function against their inducing agents; these are found to be quite heterogeneous. Thus, a bacterial product (LPS), a virus (Sendai virus), a T cell mitogenic agent (PHA), and tumour cells have all been found to induce TNF. Like the IFNs, CTXs may, therefore, play a rather non-specific role in the immune response, contributing to the defence against a variety of pathogenic agents. Indeed, one may infer a heterogeneity of functions for CTXs from the way they affect cells: a role for TNF in the immune response to viral infection may be indicated by the increased vulnerability to its cytotoxic effect in cells infected by certain viruses. An antibacterial role is perhaps indicated by the augmentation of phagocytic activity of granulocytes by TNF and an anti-tumour role by its cytotoxic effect against certain tumour cell lines.

(2) Some of the leukocyte-mediated cytotoxic activities (such as that of the cytotoxic T lymphocytes) are found to be exerted solely against those target cells to which these leukocytes bind. Involvement of CTXs in such "contact-dependent" cytotoxic activities has often been suggested, but remains to be confirmed. Even if it turns out that TNF or LT do take part, it is unlikely to be their sole function. The fact that those proteins can be induced by both cellular and non-cellular inducers (such as LPS) and the way the CTXs function, by binding avidly to cell surface receptors, which should allow them to affect remote target cells, strongly suggest that TNF and LT have a role as mediators which function distantly from their producing cells.

(3) We might understand the function of CTXs better if we bear in mind that these proteins are likely to be produced in areas of inflammation; specifically in inflammation reflected in delayed-type hypersensitivity (DTH). Indeed, among the T lymphocytes, the ones believed to be most involved in DTH (the Lyt 1^+ cells) are those found to produce the CTXs (Cardley *et al.*, 1980; Tite *et al.*, 1985). In the micro-environment formed by "walling off" the inflammatory area as a result of lymphatic blockage, CTXs may function more effectively. They may reach a high concentration

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along with IFNs produced *in situ* which can potentiate the actions of the CTXs. The cytotoxic functions of the CTXs, found to be greatly increased at elevated temperatures *in vitro* (Peter *et al.*, 1973; Ruff and Gifford, 1981b) can be potentiated by the fever in the area of inflammation. Limited supply of nutrients and of oxygen to this region may sensitize cells to the cytotoxic effect of CTXs, similarly to the sensitization observed *in vitro* when applying metabolic blockers to cells. Accumulation of MP and of granulocytes at the site of delayed type hypersensitivity, where CTXs are likely to be formed, should allow these cells to respond to the immunoregulatory effects of the CTXs. In addition, response of remote tissues to the CTXs leaking into the circulation from the site of inflammation, such as the inhibition of lipoprotein-lipase by TNF, may contribute to the defence at the level of the whole organism. Unfortunately, most information on the role of DTH reactions relates to the pathological implications of this inflammatory response (tissue damage caused in viral infection, graft rejection, etc.). This information may provide clues to the nature of situations in which CTX formation is deleterious, but gives little idea of what positive functions the CTXs can fulfil.

(4) Although the function of CTXs is not antigen-specific, there does appear to be some selectivity in their effects. That selectivity is dictated firstly by dependence of the function of CTXs (at least the function of TNF and LT) on cell surface receptors to those proteins. While the amounts of these receptors are known to vary, there is no information yet on the physiological parameters that control the variation, other than the fact that some increase in the receptor level can be induced by IFN. (As mentioned above (see page 113) we have recently noted that TNF receptors are subjected also to effective modulation by interleukin-1. Unlike the IFN-induced increase in TNF receptors which appears to reflect increased synthesis of the receptor protein, the IL-1 induced decrease in receptors for TNF is independent of protein synthesis, and probably reflects uptake or inactivation of the receptors (Holtmann and Wallach, submitted).) Further selectivity may be imposed on the function of CTXs by the dependence of their cytotoxic effect on a prior sensitization of the affected cell. Although the biochemical nature of the sensitization mechanism is not known, it appears, by the nature of those agents known to sensitize cells (metabolic blockers, viruses and x-radiation), that it is due to interference with some vital functions of the cell. CTXs can thus be viewed as agents with the capacity for distinguishing between "healthy" cells, which can resist their cytotoxicity and thus be spared from killing, and "sick" cells, which fail to resist CTX cytotoxicity and are therefore specifically eliminated.

(5) At present, there is no solid evidence for a specific anti-tumour role of CTXs, linking vulnerability of the tumour cell to CTXs with a specific

characteristic of malignancy—for exampl
dently of supply of growth-factors or the a
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VI CONCLUDIN

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characteristic of malignancy—for example, the growth of the cells independently of supply of growth-factors or the activation of specific oncogenes. Yet the mere fact that some kinds of tumour cells respond to the cytotoxic or cytostatic effects of CTXs, even though they have failed to respond to mechanisms that normally restrain cell growth, is potentially applicable in tumour therapy (see Rundell and Evans, 1981; Ruff and Gifford, 1981a; Lee *et al.*, 1984). Perhaps it will be possible to direct CTX activity selectively against tumour cells, taking advantage of the fact that DNA-intercalating drugs and ionizing radiation, which preferentially affect rapidly-growing tumour cells, sensitize cells to the cytotoxic effect of CTXs. From the point of view of IFN research, the most intriguing question is whether effects of CTXs on tumour cells and their synergism with IFN can contribute to increasing the effectiveness of IFN in tumour therapy: do CTXs produced *in situ*, formed in increased amounts owing to the enhancement of CTX formation by IFN, contribute to some of the anti-tumour effects that IFNs have already been found to exert, and can CTXs, injected into patients together with IFN, further potentiate these anti-tumour effects?

VI CONCLUDING REMARKS

While there is some recent information on the structure of CTXs, our knowledge of their function, the mechanisms involved, and their physiological role is still quite limited. Basically what has been learned in the short time that purified CTXs have been available is that the way in which CTXs function is more complex than was believed hitherto. The cytotoxic activity of the CTXs had been thought to be non-specific, leading to killing of "innocent bystander cells". A more thorough examination now shows that this activity can be subjected to effective modulation, depending on the nature of the target cell and its metabolic state. Furthermore, when they do not exert cytotoxic effects, CTXs mediate other regulatory functions.

Which of the various effects of CTX function observed *in vitro* leads to physiologically meaningful consequences is not known. Nor can we tell yet whether the practical benefits of these proteins, such as the killing of tumour cells synergistically with IFN, will be found sufficiently effective to be applicable in therapy. Perhaps it will be the adverse effects, such as mediation of cachexia or of shock symptoms in disease, that will turn out to be the more prominent, necessitating a search for means of suppressing CTX activity. We know that TNF and LT do not act simply by exerting an enzymatic activity but rather by binding to specific receptors, thus activating cellular mechanisms that affect gene regulation and can also mediate cell death. The nature of those mechanisms is yet to be elucidated. Knowledge of the mechanisms

initiating synthesis of CTXs within the CTX-producing cells is also quite limited.

Our present incomplete state of knowledge of the CTXs provides intriguing hints toward a deeper understanding in the future. The lack of resemblance between the TNFs (TNF and LT) and other known proteins; the way these proteins kill cells; the presence of receptors to TNF and LT on many differing cells; the ability of TNF and LT to initiate cellular responses other than cell death; and the multiplicity of agents that induce production of these proteins, all indicate that further studies on these proteins, as well as of the way their formation and function are regulated by IFN, may disclose novel information on mechanisms controlling the life and death of cells and on ways whereby the immune response can regulate these mechanisms.

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Two Different Cell Types Have Different Major Receptors for Human Tumor Necrosis Factor (TNFα)*

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The receptors for tumor necrosis factor α (TNFα) were analyzed on myeloid cells (HL60, U937, K562, and freshly isolated blood monocytes) and on cells of epithelial origin (MCF7, HEp2 and HeLa cells), by use of radiolabeled TNFα and cross-linking experiments. Both cell types had high but slightly different affinities for TNFα. The myeloid cells had major cross-linked products of 98–100 kDa, which were similar in their *N*-linked glycosylation, whereas the cells of epithelial origin contained a major cross-linked product of 75 kDa, a second product of 95 kDa. The major receptors of both cell types (studied mostly with HL60 and HEp2 cells) are different proteins because (*a*) their apparent molecular masses were different and no evidence was obtained for cell-specific proteases, which could generate the differently sized receptors from one common receptor molecule; (*b*) anti-receptor antibodies, which precipitated the 95- and 75-kDa products, did not precipitate the 100-kDa cross-linked complex; (*c*) the native TNFα-receptor complexes had different proteolytic fingerprints; (*d*) the tryptic fragments differed in their association with the cell membrane vesicles; (*e*) the receptors differed in their degree of *N*-linked glycosylation; and (*f*) *O*-linked glycosylation was found on the major receptor of HL60 but not of HEp2 cells. In addition, myeloid cells may also contain a small amount of the HEp2-type of TNFα receptor. We suggest that at least two different receptors for TNFα exist.

Tumor necrosis factor α (TNFα)[1] is a protein released by activated macrophages in response to external stimuli. It is implicated in a wide variety of human diseases (for review, see Ref. 1), may be effective against certain cancers, plays a role in septic shock and cachexia (for reviews, see Refs. 2 and 3), is a mediator of inflammation and of various immunological reactions (for reviews, see Refs. 4–6), and also seems to be important in some pathological effects seen in malaria (7–9).

TNFα was originally characterized as a protein, which induces necrosis in certain tumors (10). Later it was recognized to be a cytokine with diverse effects on different tissues and cells, *in vivo* as well as *in vitro* (for review, see Ref. 4). Among these effects are (*a*) the activation of the endothelium, which leads to release of platelet-derived growth factor (11) and to changes in the endothelial morphology and cellular organization (12); (*b*) the stimulation of human B-cells, if TNFα is combined with other B-cell mitogens (13); and (*c*) other immunomodulatory properties (14). The pleiotropic biological effects of TNFα are frequently enhanced synergistically by interferon-γ (15) or, in the case of hemorrhagic necrosis and lethal shock, by bacterial lipopolysaccharides (16). The release of TNFα can be inhibited by transforming growth factor-β (17).

The majority of human cells have specific and high affinity binding sites for TNFα (18, 19). These surface receptors are believed to mediate the cellular effects of TNFα. Anti-TNFα antibodies, which inhibit the binding of TNFα to the cell, also block the biological effects of TNFα (20). In addition, a decrease in the number of cell surface receptors correlated with a decreased sensitivity to the cytolytic effect of TNFα in certain cells (21, 22), and half-maximal release of interleukin-1 by endothelial cells was seen with the same concentration of TNFα that showed half-maximal binding to the cells (23). However, binding of TNFα to a receptor and internalization of the receptor-ligand complex into the cell is not sufficient for the cytotoxicity of TNFα, because cells resistant to TNFα can also have TNFα receptors and internalize the bound TNFα molecules (24).

Cross-linking of radiolabeled TNFα to its cellular receptor has been used by several groups to study the TNFα receptors. Different sizes of cross-linked products were reported for different cell types: 95- and 75-kDa products for the mouse fibroblast cell line L-M (CCL 1.2) (18); 92 and 75 kDa for U937 (histiocytic lymphoma), KG-1 (promyelocytic leukemia), and FS-11 (normal foreskin fibroblast) cells (25); 100 kDa for U937 cells (26); 138, 90, 75, and 54 kDa for MCF7 cells (27); and 102 kDa for monocytes (28). It is unclear what the relation is between the different cross-linked products.

In this paper, we compare the TNFα receptors on different cell types. Myeloid cells showed major cross-linked products of 98–100 kDa, whereas the major cross-linked products of cells of epithelial origin were 75 and 95 kDa. We show that the major receptors of both cell types differ in their native structure and that the differences in apparent molecular masses within the myeloid group may be solely the result of differences in receptor glycosylation.

MATERIALS AND METHODS

Cells—The following cell lines were used: the human cell lines HL60, HEp2, MCF7, HeLa, U937, and K562. The cell lines were obtained from ATCC and maintained over many passages in RPMI 1640 medium, supplemented with 10% (v/v) fetal calf serum or 10% (v/v) horse serum. The adherent cells (HEp2, MCF7, and HeLa cells) were grown to confluency. The nonadherent cells (HL60, U937, and K562 cells) were harvested after reaching a density of 1–1.5 × 10^6 cells per ml of culture medium. Human peripheral blood monocytes were isolated from the blood of healthy individuals (29). For inhibition

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[1] The abbreviations used are: TNF, tumor necrosis factor; PMSF, phenylmethanesulfonyl fluoride; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

of *N*-glycosylation, tunicamycin (Boehringer Mannheim) was added to 1–2 μg/ml of culture medium (myeloid cells 1 μg/ml; HEp2 cells 2 μg/ml) 16 h before harvesting the cells. For tunicamycin experiments with monocytes, the cells were resuspened in culture medium to a density of 1×10^6 cells/ml, tunicamycin was added, and cells were incubated for 16–20 h at 37 °C in 50-ml Falcon centrifuge tubes.

TNFα Labeling and Cross-linking—Human TNFα was purified from recombinant *Escherichia coli* cells. The protein was purified by ion-exchange chromatography on DEAE-Sepharose and high pressure liquid chromatography using TSK-3000 columns (Pharmacia LKB Biotechnology Inc.) (30). The purified TNFα was more than 99% homogeneous, as judged by SDS-PAGE and Coomassie Blue staining, and had a cytotoxic activity on WEHI-164 cells of 5×10^7 units/mg TNFα. The protein was radio-iodinated with Iodogen (Pierce Chemical Co.), according to Ref. 31, and purified over a PD10 column (Pharmacia LKB Biotechnology Inc.). The radiolabeled protein was homogeneous, as judged by SDS-PAGE and autoradiography, and had a specific activity of about 500 Ci/mM of TNFα. The amount of radiolabeled TNFα was measured using a sandwich immunoassay.

Nonadherent cells were harvested by centrifugation for 5–10 min at 1500 rpm (about 300 × *g*); the cells were resuspended in RPMI 1640 medium supplemented with 10% fetal calf serum, 20 mM HEPES-NaOH (pH 7.2), and 0.1% (w/v) sodium azide (incubation medium) and washed once by centrifugation. The cells were then resuspended at a density of 0.5–5 × 10^7 cells per ml of incubation medium supplemented with 3 nM radiolabeled TNFα. The cells were gently agitated during the incubation with the radiolabeled TNFα to prevent sedimentation. Where indicated, the incubations were done in the presence of 200 nM unlabeled TNFα. After 2 h on ice, the cells were collected by centrifugation, washed three times with PBS (10 mM sodium phosphate (pH 7.2), 150 mM NaCl), and resuspended in PBS to 0.5–5 × 10^7 cells per ml. The cross-linker bis(sulfosuccinimidyl)suberate (Pierce Chemical Co.) was added to 1 mM final concentration from a freshly prepared 100 mM stock solution in water. After incubation for 20 min on ice, the cells were centrifuged and incubated for 10–30 min in PBS supplemented with 20 mM glycin to inactivate the remaining cross-linker. The cells were again collected, and the amount of radiolabeled TNFα bound to the cells was determined using a γ-counter, and the cell pellets were then stored frozen.

The adherent cells were grown until they reached confluency. The culture medium was removed, and the cells were then rinsed once with incubation medium. The cells were incubated on ice for 2 h with 3 nM radiolabeled TNFα. The incubation medium was then removed; the cells were rinsed three times with PBS and incubated with the cross-linker as described above. The cells were then scraped from the tissue culture flask using a rubber policeman, analyzed for bound radiolabeled TNFα, and the cell pellets stored frozen. Identical results were obtained if the adherent cells were incubated with the radiolabeled TNFα and cross-linked in suspension after removal from tissue culture flasks by incubation with 1 mM EDTA at 37 °C for 5 min. Incubation of HL60 and HEp2 cells with radiolabeled TNFα for the Scatchard analysis was performed in suspension.

The cell pellets were thawed and resuspended to a density of 0.5–5 × 10^7 cell equivalents per ml of PBS containing the protease inhibitors benzamidine and PMSF (10 and 1 mM final concentrations, respectively). In some cases, the protease inhibitors were omitted (see below). The cell suspensions were sonicated using a cup horn until complete cell lysis was obtained. A 1500 × *g* supernatant was prepared. The pellet, which contained 5–10% of the total amount of radioactivity bound to the cells, was discarded. In the experiment described in Fig. 2 the cells were lysed by the addition of SDS-containing sample buffer, and cellular DNA was fragmented by sonication.

Trypsin Digestion—1,500 × *g* supernatants of sonicated cells without added protease inhibitors were incubated for the indicated times at 37 °C with 0.03–1 mg of trypsin (N^a-*p*-tosyl-L-lysine chloromethyl ketone-treated, Boehringer Mannheim) per ml of incubation medium. The reactions were stopped by heating the samples to 95 °C and addition of SDS-containing gel sample buffer. To investigate the membrane association of tryptic fragments, 1,500 × *g* supernatants of sonicated cells were incubated for 1 h at 37 °C with 0.1 mg of trypsin per ml. The protease digestion was stopped by cooling to 0 °C, addition of soybean trypsin inhibitor (Boehringer Mannheim) to 0.2 mg/ml and of benzamidine and PMSF to 10 and 1 mM, respectively. The samples were centrifuged in a Beckman TL100 tabletop ultracentrifuge for 10 min at 350,000 × *g*. The supernatants were collected; the pellets were resuspended in PBS, and SDS-containing gel sample buffer was added.

Enzymatic Deglycosylation—1,500 × *g* supernatants of sonicated HL60 cells were incubated at 37 °C with 1 mg of trypsin/ml. After 1 h, 2 mg of soybean trypsin inhibitor was added per ml of incubation medium. The trypsin-digested and untreated 1,500 × *g* supernatants were adjusted to 0.2% (w/v) SDS, 1% (v/v) 2-mercaptoethanol, and 10 mM EDTA. After 30 min incubation on ice, Triton X-100 (Fluka) was added to 0.6% (v/v), and the samples were boiled for 1 min. PMSF (1 mM) and benzamidine (10 mM) were added, and the samples were incubated overnight at 37 °C with 20 units of *N*-glycanase (Boehringer Mannheim) per ml. For incubation with neuraminidase and *O*-glycanase, 1,500 × *g* supernatants of sonicated HEp2 and HL60 cells were centrifuged in a Beckman TL100 tabletop ultracentrifuge for 15 min at 200,000 × *g*. The pellets were resuspended in 20 mM Tris maleate (pH 6), to 3 × 10^6 cell equivalents per ml and 60 μl of these suspensions were incubated with 35 milliunits of neuraminidase (Behring Diagnostics) and/or 1.5 milliunits of *O*-glycanase (Boehringer Mannheim), according to manufacturers' instructions.

Immunoprecipitations—HEp2 and HL60 cells were incubated with TNFα and cross-linker and were sonicated; 1,500 × *g* supernatants were made and then centrifuged in a Beckman TL100 tabletop ultracentrifuge for 15 min at 200,000 × *g*. The pellets were resuspended in RIPA buffer (10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 1% deoxycholate, 0.1% SDS, 0.25 mM PMSF) to 2 × 10^8 cells per ml, and insoluble material was removed by ultracentrifugation after 30 min of incubation on ice. 5 μg of monoclonal antibody was added to 50 μl of detergent extract. The immunocomplexes were incubated overnight at 4 °C and then recovered by addition of 20 μl of RIPA buffer with 4 μl of Sepharose beads coated with protein G (Pharmacia LKB Biotechnology Inc.).

Gel Electrophoresis and Autoradiography—SDS-PAGE was carried out according to Ref. 32 in 7.5–15% isokinetic gradient gels. After the run, the gels were silver-stained (33), dried, and exposed at −70 °C to Kodak XAR-5 films using Quanta III intensifying screens.

RESULTS

Different Sizes of Cross-linked TNFα-TNF Receptor Complexes Are Found on Various Cell Types—Cross-linking experiments were carried out to visualize TNFα receptors on different cells. Two different cell types were used: myeloid cells (HL60, U937, K562, and freshly isolated blood monocytes) and cells of epithelial origin (MCF7, HEp2, and HeLa cells). Cell suspensions were incubated with radiolabeled TNFα at 0 °C in the presence of sodium azide to prevent internalization of the receptor-ligand complexes. The incubations were done in the presence or absence of an excess of unlabeled TNFα to determine the specificity of the binding. The complexes were then chemically cross-linked under mild cross-linking conditions. The cells were pelleted, washed, and the labeled proteins analyzed by SDS-PAGE. Cross-linked dimeric and trimeric forms of the radiolabeled TNFα were seen if the purified and radiolabeled TNFα was incubated with the cross-linker in the absence of cells (Fig. 1, *lanes 1* and *2*). Additional cross-linked products were seen if radiolabeled TNFα was incubated with cells prior to incubation with the cross-linker (Fig. 1, *lanes 3–16*). The myeloid cells had major cross-linked products with apparent molecular masses of 98–100 kDa (Fig. 1, *lanes 3–10*), although some minor bands were also seen. A different cross-linking pattern was seen if cells of epithelial origin were used. The major cross-linked products of these cells had apparent molecular masses of 95 and 75 kDa, the 75-kDa protein being the most abundant cross-linked product (Fig. 1, *lanes 11–16*). Some of the minor cross-linked products of the myeloid cells co-migrated with the major bands seen with the cells of epithelial origin. The ratio between the 100-kDa product and these minor bands was not constant in different experiments (see also Fig. 3). Our data suggest that these minor bands are not identical to the major bands seen in HEp2 cells (see below). No additional cross-linked products were seen under other

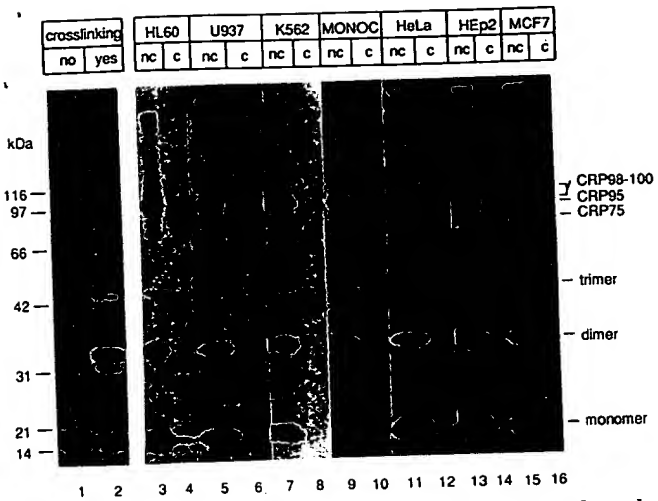
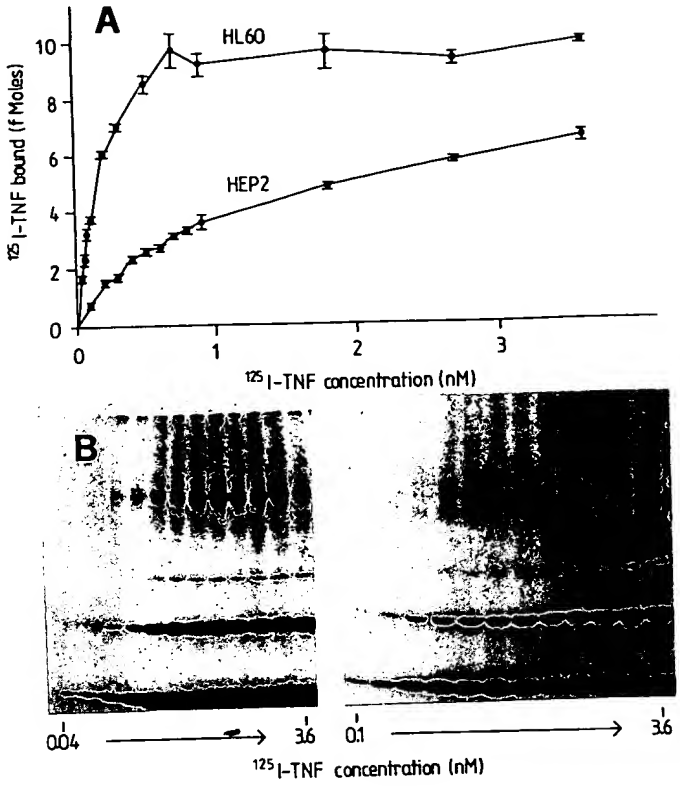


FIG. 1. **Two different patterns of cross-linked products between radiolabeled TNFα and cell surface proteins in different cell types.** *Lanes 1* and 2, purified radiolabeled TNFα was incubated without (*lane 1*) or with (*lane 2*) cross-linker. *Lanes 3–16,* cross-linking of radiolabeled TNFα to the indicated cells, in the absence (*nc,* not competed) or presence (c, competed) of an excess unlabeled TNFα. After cross-linking, the cells were lysed with detergent and 1500 × *g* supernatants corresponding to 1–2 × 10^6 cell equivalents were analyzed by SDS-PAGE and autoradiography. Myeloid cells (*lanes 3–10*) and cells of epithelial origin (*lanes 11–16*) were used. The positions of the different cross-linked products (*CRP,* cross-linked product) and of the monomer, dimer, and trimer of TNFα are indicated. *Monoc,* human blood monocytes.



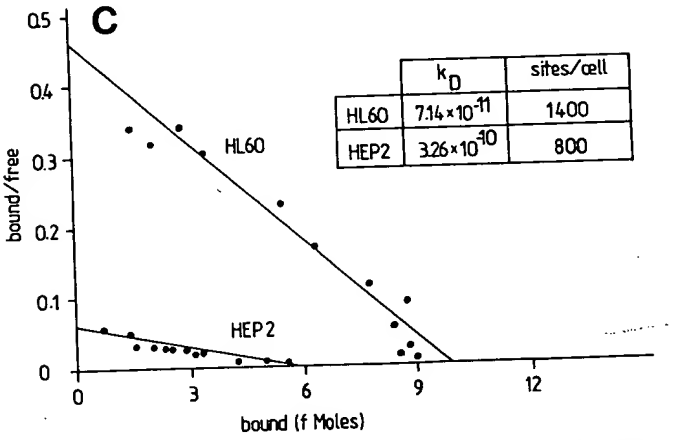


FIG. 2. **Scatchard analysis of the binding of radiolabeled TNFα to HL60 cells and HEp2 cells.** HL60 or HEp2 cells (1 × 10^6 cells per assay, in triplicate) were incubated with increasing concentrations of radiolabeled TNFα, in absence or presence of unlabeled TNFα. The cells were washed, incubated with cross-linker, and the amount of the radiolabeled TNFα specifically bound to the cells (the difference between the total amount of radioactivity bound in the absence and in the presence of excess unlabeled TNFα) was determined (*panel A*). The amount of radioactivity bound to the cells in the presence of excess unlabeled TNFα was always less than 10% the amount bound in the absence of unlabeled TNFα. The mean of specific binding ± S.E. of total binding is indicated. After counting the bound radioactivity, the cells were lysed by addition of SDS-containing gel sample buffer and analyzed by SDS-PAGE and autoradiography (*panel B*). Analysis of the binding data and curve fitting according to Scatchard was performed using the computer program "Ligand," revised version 2.3.11 (37) (*panel C*). The number of binding sites was calculated assuming that one TNFα trimer binds to one receptor molecule.

cross-linking conditions (except for high molecular weight aggregates which did not enter the gel) nor if cross-linkers with other molecular spans were used (data not shown).

The Major TNFα Receptors of HL60 Cells and HEp2 Cells Have High but Slightly Different Affinities for Radiolabeled TNFα: Scatchard Analysis—Scatchard analysis was carried out to determine the affinities of the TNFα receptors on HL60 and HEp2 cells. The binding curves are shown in Fig. 2*A*. As a control, the cells were cross-linked to visualize the radiolabeled TNFα-receptor complexes. The patterns (Fig. 2*B*) were independent of the concentration of radiolabeled TNFα, whereas the amounts of the cross-linked products increased with increasing TNFα concentration. One type of binding site per cell type was detected in the Scatchard analysis (Fig. 2*C*). The calculated affinities (K_d values) were 7.14×10^{-11} for HL60 cells and 3.26×10^{-10} for HEp2 cells. The calculated number of receptors per cell was 1400 for HL60 cells and 800 for HEp2 cells, assuming that TNFα binds as a trimer to its receptor (34, 35). For HL60 cells, the calculated affinity may correspond to that of the major site detected in the cross-linking experiments, since the amount of the cross-linked products increased similarly to the increase in the total amount of radiolabeled TNFα bound to HL60 cells (Fig. 2*B, left panel*). For HEp2 cells, the amount of cross-linked products also increased similarly to the increase in the total amount of TNFα bound to the HEp2 cells, but multiple cross-linked products were seen (Fig. 2*B, right panel*).

No Cell-specific Proteases Were Found Which Could Generate the Different Cross-linked Products of HEp2/MCF7 Cells from the HL60 Type Receptor—Experiments were done to determine whether the TNFα receptor of HEp2 cells was a proteolytic product of the HL60 type of receptor.

To test for proteases located on the HEp2 cell surface, HEp2 and HL60 cells were mixed directly after harvesting. The mixtures were then incubated with radiolabeled TNFα and processed as described. Fig. 3 (*lanes 1–4*) shows that the ratios of the cross-linked bands in the mixing experiments were only dependent on the mixing ratios of both cells. Similar results were obtained if the mixing experiments were performed with MCF7 cells (data not shown). Thus, the different cross-linked patterns were not due to protease digestion by a

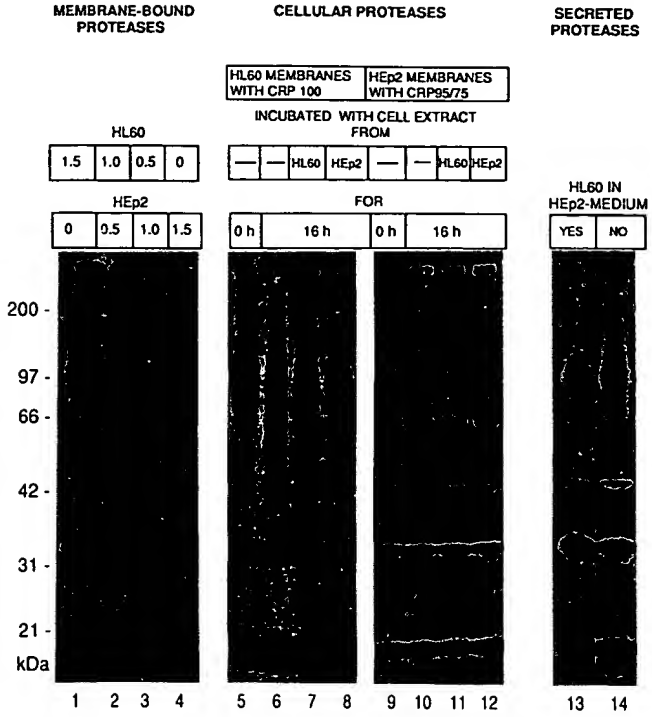


FIG. 3. **The cross-linked products of the HEp2 type of TNFα receptor may not be generated by proteolysis of the 100-kDa type of cross-linked product found on HL60 cells.** *Lanes 1–4,* testing for membrane-bound proteases, which could degrade the 100-kDa cross-linked product after harvesting the cells. HL60 cells and HEp2 cells were harvested and mixed together in various ratios as indicated. The cells were then incubated with radiolabeled TNFα and cross-linker, and 1500 × *g* supernatants of cell lysates were prepared. *Lanes 5–12,* testing for differences in intracellular proteases. 1500 × *g* supernatants of cell lysates containing cross-linked products, each corresponding to 1 × 10^6 cells, were incubated at 37 °C, eventually after addition of a 1500 × *g* supernatant of cell lysates of 1 × 10^6 cells, not incubated with TNFα and cross-linker. *Lanes 13* and *14,* testing for proteases, possibly released by HEp2 cells into culture medium. HL60 cells were harvested and resuspended to 0.8 × 10^6 cells per ml, either in a mixture of 75% spent culture medium of HEp2 cells and 25% (v/v) fresh medium (*lane 13*) or in fresh medium only (*lane 14*). After 16 h the cells were harvested and incubated with radiolabeled TNFα and cross-linker. Cell extracts equivalent to 1 × 10^6 cells were then made. All samples were analyzed by SDS-PAGE and autoradiography.

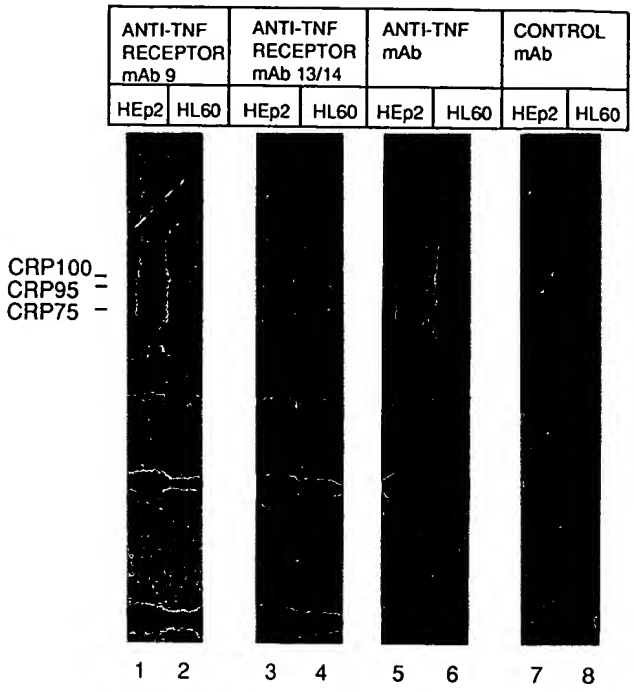


FIG. 4. **Monoclonal antibodies against a TNFα receptor, which precipitate the cross-linked products of HEp2 cells, do not precipitate the major cross-linked product of HL60 cells.** Detergent extracts of HEp2 or HL60 cells containing cross-linked products (*CRP*) were incubated with monoclonal antibodies (*mAb*) against a TNFα receptor (*lanes 1–4*), against TNFα itself (*lanes 5* and *6*), or with an unrelated control antibody. The immunocomplexes were collected and analyzed by SDS-PAGE and autoradiography.

cell-specific and membrane-bound protease after the harvesting of the cells. In addition, this also excluded the possibility that the different cross-linked patterns were the result of potential differences in the cross-linking conditions used for both cell types.

To test the intracellular proteases, HL60 cells (Fig. 3, *lanes 5–8*) and HEp2 cells (Fig. 3, *lanes 9–12*) were incubated with radiolabeled TNFα and cross-linker, were lysed by sonication, and 1500 × *g* supernatants were made. These supernatants were then directly frozen (Fig. 3, *lanes 5* and *9*) or were incubated for 22 h at 37 °C either without any addition (Fig. 3, *lanes 6* and *10*) or after addition of unlabeled extracts of either HL60 cells (Fig. 3, *lanes 7* and *11*) or HEp2 cells (Fig. 3, *lanes 8* and *12*). Proteolytic degradation of the cross-linked products of HL60 cells was seen (compare Fig. 3, *lanes 6–8* with *lane 5*), whereas no digestion of the cross-linked products of HEp2 cells was seen (compare Fig. 3, *lanes 10–12* with *lane 9*). However, no differences were found between the digestion patterns after addition of HL60 extract or HEp2 extract (compare Fig. 3, *lanes 7* and *8*). The digestion products of HL60 cells (Fig. 3, *lanes 6–8*) were also smaller than the cross-linked products of HEp2 cells (Fig. 3, *lanes 9–12*). Thus, *no* HEp2-specific intracellular protease was detected, which could convert the HL60 type of TNFα receptor into the HEp2 type.

To test for specific proteases, eventually released by HEp2 cells into the culture medium, HL60 cells were incubated for 16 h either in 75% spent medium from HEp2 cells (Fig. 3, *lane 13*) or in fresh culture medium (Fig. 3, *lane 14*). After the incubation, the HL60 cells were incubated with radiolabeled TNFα and cross-linker and were analyzed by SDS-PAGE. *No* evidence was obtained for a protease, secreted by the HEp2 cells into the conditioned medium, which could convert the HL60 receptor type into the HEp2 type.

In summary, *no* evidence was obtained for a protease, produced by HEp2 cells, which could convert the major TNFα receptor of HL60 cells into proteins with molecular masses comparable with those of the major TNFα receptors of HEp2 cells.

Antibodies against a TNFα Receptor Immunoprecipitate the 75- and 95-kDa Cross-linked Bands of HEp2 Cells but Do Not Precipitate the 100-kDa Cross-linked Complex from HL60 Cells—Monoclonal antibodies were made against purified TNFα receptor protein.[2] The antibodies were tested for immunoprecipitation of the cross-linked complexes of HEp2 and HL60 cells. Antibodies mAb9 and mAb13/14 precipitated the 75- and 95-kDa cross-linked products of HEp2 cells (Fig. 4, *lanes 1* and *3*) but not the 100-kDa cross-linked product of HL60 cells (Fig. 4, *lanes 2* and *4*). In addition, the minor 75-kDa cross-linked product of HL60 cells was also not immunoprecipitated with the anti-receptor antibodies, suggesting that this minor 75-kDa cross-linked product of HL60 cells is not identical to the major 75-kDa cross-linked band in HEp2 cells (Fig. 4). A fraction of the TNFα molecules, which was

[2] M. Brockhaus and H.-R. Lötscher, manuscript in preparation.

not cross-linked to the receptor, was also precipitated by the TNFα receptor antibodies. This indicated that this fraction of the TNFα was co-precipitated together with the receptor protein, since the antibodies were shown not to cross-react with TNFα itself.[2] Thus, at least a fraction of the receptor-ligand complexes remained intact during the immunoprecipitation procedure and was only dissociated upon SDS-PAGE (Fig. 4, *lanes 1–4*). Antibodies against TNFα itself immunoprecipitated both the cross-linked products of HEp2 cells and of HL60 cells (Fig. 4, *lanes 5* and *6*), whereas no precipitation was seen if an unrelated control antibody was used (Fig. 4, *lanes 7* and *8*). The inability to immunoprecipitate the 100-kDa cross-linked product of HL60 cells is not the result of extensive glycosylation of this receptor protein, because no precipitation was seen if *N*- and *O*-linked sugars were removed from the 100-kDa product (see below) before immunoprecipitation (not shown). A small fraction of the noncross-linked TNFα was co-precipitated from HL60 extracts with the anti-receptor antibodies (Fig. 4, *lanes 2* and *4*). However, no discrete cross-linked complexes between TNFα and receptor proteins were visible in the immunoprecipitates, only a smear in the 75–100-kDa region of the gel. This suggested that HL60 cells, in addition to the 100-kDa cross-linked product, also have a minor receptor population, of undefined apparent molecular mass, which contains the epitope recognized by the anti-receptor antibodies.

The Extracellular Domains of the Major TNFα Receptors on HL60 Cells and HEp2 Cells Differ in Their Sensitivity to Protease Digestion—Proteolytic fingerprints were made to characterize the extracellular domains of the major TNFα receptors on HL60 cells and HEp2 cells. Cells were incubated with radiolabeled TNFα and cross-linker, sonicated, and then incubated with trypsin. The digestions were stopped and the samples analyzed by SDS-PAGE. The major cross-linked bands of 75 and 95 kDa (CRP 75 and CRP 95) of HEp2 cells (Fig. 5*A*, *lane 1*) were already digested at the lowest trypsin concentration used (Fig. 5*A*, *lane 9*). A digestion intermediate of 54 kDa (tryptic fragment 54) and a relatively stable digestion product of 45 kDa were found (tryptic fragment 45) (Fig. 5*A*, *odd lanes*). The major cross-linked product of HL60 cells (CRP 100) (Fig. 5*A*, *lane 2*) was much more resistant to trypsin digestion. The 100-kDa cross-linked product was converted via a putative intermediate of about 75 kDa (tryptic fragment 75) into a relatively stable digestion product of about 48 kDa (tryptic fragment 48) (Fig. 5*A*, *even lanes*). The 75-kDa digestion intermediate of HL60 cells (tryptic fragment 75) has a slightly different size compared with the 75-kDa cross-linked product of HEp2 cells. Both proteins are, however, very different in their sensitivity to trypsin digestion, suggesting different native structures of these proteins. Little digestion of the noncross-linked radiolabeled TNFα molecules was seen under the digestion conditions used (Fig. 5*B*). These TNFα molecules were bound to the receptor during the digestion, as suggested from their co-precipitation with anti-receptor antibodies (Fig. 4). The amount of the dimer of TNFα was, however, drastically reduced. The exact reason for this reduction is not known (Fig. 5*A*).

The trypsin digestion products of HL60 and U937 cells were not bound to membranes; the fragment was recovered in a 350,000 × *g* supernatant (Fig. 6, *lanes 7* and *11*) and, thus, represents (part of) the receptor's extracellular domain. In contrast, most of the digestion products of HEp2 cells were recovered in the 350,000 × *g* pellet (Fig. 6, *lane 4*).

Thus, the tryptic fingerprints of the native TNFα receptors on HL60/U937 cells and HEp2 cells were different and the

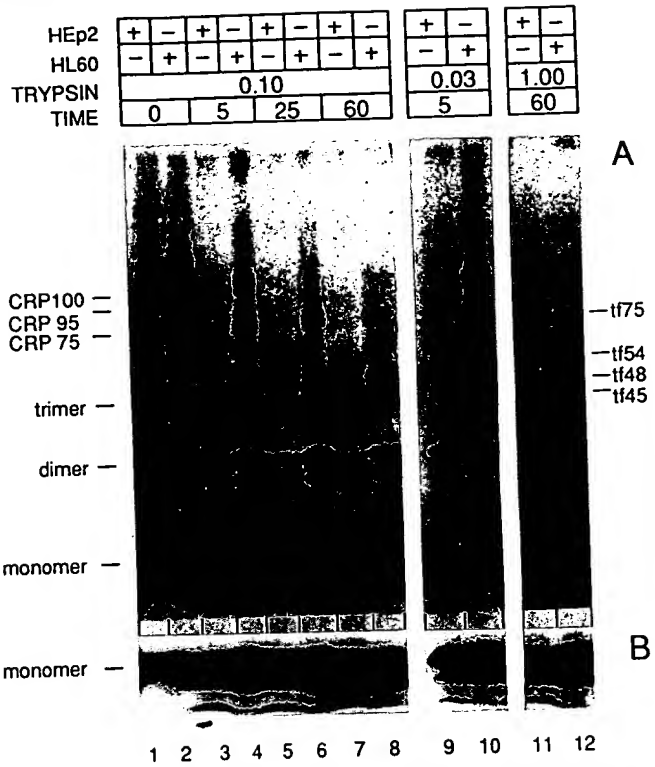


FIG. 5. **The cross-linked products of HL60 and HEp2 cells show different native tryptic fingerprints.** HL60 and HEp2 cells were incubated with radiolabeled TNFα and cross-linker, were lysed by sonication, and 1500 × *g* supernatants were prepared. Aliquots, each containing 5000 cpm, were incubated for 5–60 min with 0.03–1 mg of trypsin per ml of incubation medium. The samples were then analyzed by SDS-PAGE and autoradiography (*panel A*). *Panel B*, short exposure of the lower part of the gel of *panel A*. The positions of the intact cross-linked products (*CRP*) of the monomer, dimer, and trimer of TNFα and of the most abundant tryptic fragments (*tf*) are indicated.

fragments differed in their association with the membrane vesicles.

The Major TNFα Receptors on HL60 and HEp2 Cells Differ in Their N-Linked Glycosylation—Cells were grown in the presence or absence of tunicamycin (an inhibitor of *N*-linked glycosylation), incubated with radiolabeled TNFα and cross-linker, and processed as described. A cross-linked product of 98 kDa was seen if extracts of HL60 cells grown without tunicamycin were incubated with *N*-glycanase (Fig. 7, *lanes 1* and *2*). In tunicamycin-grown cells, two cross-linked products of 98 and 96 kDa were found (Fig. 7, *lanes 3* and *4*). The *N*-linked sugars were found on the tryptic fragment 45 (Fig. 7, *lanes 5* and *6*, see also Fig. 5), which represents (part of) the receptor's extracellular domain (see above). The tryptic fragments of the receptor of tunicamycin-grown cells were also shorter than the corresponding *N*-glycanase fragments (Fig. 7, *lanes 6–8*). The differences between the *N*-glycanase digestion products and the cross-linked products of tunicamycin-grown cells suggested that either not all *N*-linked carbohydrates were removed by the *N*-glycanase or that the 96-kDa band is a proteolytic degradation product of the 98-kDa band.

N-Glycanase treatment of HEp2 cells led to a pronounced down-shift in the molecular masses of the cross-linked complexes (Fig. 7, *lanes 9* and *10*). Several smaller cross-linked bands were observed, which did not co-migrate with the deglycosylated products of HL60 cells (Fig. 7, compare *lane 10* with *lanes 3* and *4*). A similar down-shift was seen if cells were grown in the presence of tunicamycin (Fig. 8, *lanes 9* and *10*). The *N*-linked sugars were present on the tryptic

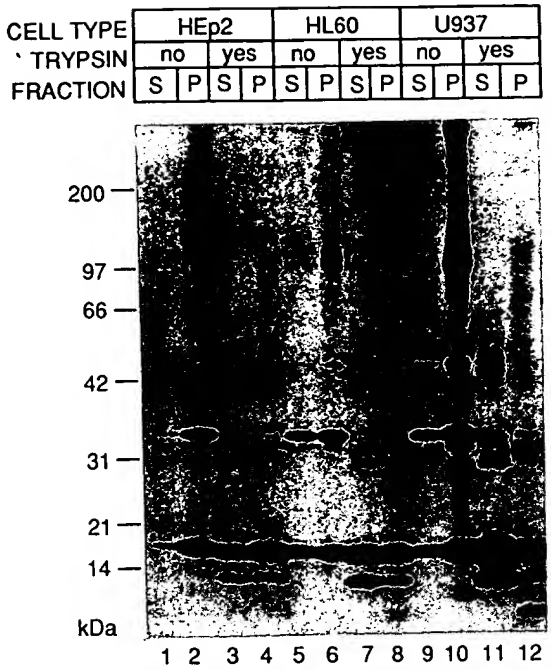


FIG. 6. **The tryptic fragments of HEp2 cells and HL60 cells or U937 cells differ in their association with cell membranes.** Cells were incubated with radiolabeled TNFα and cross-linker, were lysed by sonication, and 1,500 × *g* supernatants were prepared. The supernatants were mock-incubated or incubated for 1 h at 37 °C with trypsin. The extracts were then centrifuged for 10 min at 350,000 × *g*, and the supernatants (*S*) and pellets (*P*) were collected. Samples, each equivalent to 1.5 × 10^6 cells, were analyzed by SDS-PAGE and autoradiography.

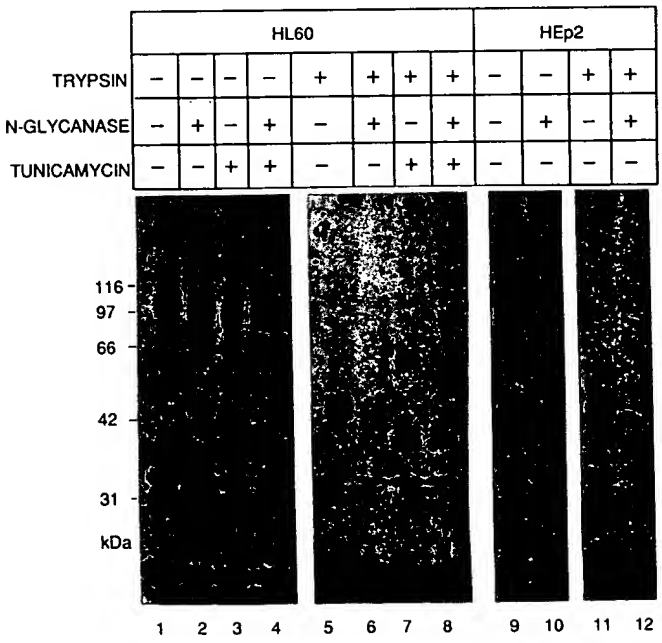


FIG. 7. **The major TNFα receptors of HL60 and HEp2 cells differ in their *N*-linked glycosylation.** Cells were grown in the absence or presence of tunicamycin, were incubated with radiolabeled TNFα and cross-linker and 1500 × *g* supernatants were prepared as described above. The supernatants were incubated, where indicated, for 15 min at 37 °C with 1 mg of trypsin per ml of incubation medium. Protease inhibitors were added, and the samples were then either mock-incubated or were incubated with *N*-glycanase. The samples were analyzed by SDS-PAGE and autoradiography.

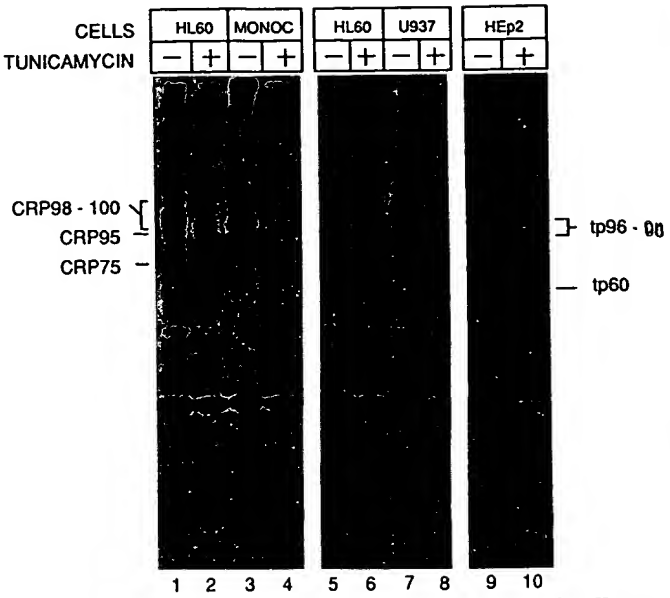


FIG. 8. **The TNFα receptors of different myeloid cells are similar in their *N*-linked glycosylation.** Cells grown in the absence (−) or presence (+) of tunicamycin were incubated with radiolabeled TNFα and cross-linker, were lysed by sonication, and 1500 × *g* supernatants were prepared. Aliquots, each containing 1.5 × 10^6 cells, were analyzed by SDS-PAGE and autoradiography. The positions of the cross-linked products of cells grown in the absence of tunicamycin (*CRP*) are indicated on the *left*; the positions of the cross-linked products of cells grown in the presence of tunicamycin (*tp*) are indicated on the *right*. *Monoc*, human blood monocytes.

fragment 45 (Fig. 7, *lanes 11* and *12*, see also Fig. 5). The binding and cross-linking of TNFα to cells grown in the presence of tunicamycin was drastically reduced in HEp2 cells (Fig. 8, *lanes 9* and *10*) but not in HL60 cells (Fig. 7, *lanes 1–8*).

Thus, the major TNFα receptors on HL60 and HEp2 cells contained *N*-linked carbohydrates, and differences in *N*-linked glycosylation do not explain the differences between the different cross-linked products.

The Major TNFα Receptors of Different Myeloid Cells Are Similar in Their N-Linked Glycosylation—The major cross-linked products found on different myeloid cells have slightly different apparent molecular masses (Fig. 1 and Fig. 8, *lanes 1, 3, 5,* and *7*). The major TNFα receptors on these cells, in contrast to those of HEp2 cells, are similar in their *N*-linked glycosylation, since similar down-shifts in the molecular masses of the cross-linked products of tunicamycin grown cells were seen (Fig. 8, *even lanes* and not shown for K562 cells).

The Major Cross-linked Products of HL60 Cells, but Not of HEp2 Cells, Contain O-Linked Glycosylation—Incubation of HL60 cells containing the 100-kDa cross-linked product with neuraminidase reduced the molecular mass of the 100-kDa product by 4–6 kDa (Fig. 9, *lanes 1* and *2*). An additional reduction in the molecular mass was seen if neuraminidase and *O*-glycanase were combined (Fig. 9, *lanes 2* and *3*). Both the reductions in molecular mass upon treatment with neuraminidase or neuraminidase plus *O*-glycanase were additive to the size reduction found in tunicamycin-grown cells (Fig. 9, *lanes 5–7*). This shows that the major HL60 receptor is *O*-linked glycosylated and that the sialic acid residues are part of the *O*-linked glycosylation. The *O*-linked glycosylation in total contributes 6–10 kDa to the receptor's molecular mass. As observed in other systems (36), the co-treatment with neuraminidase was required to see the size reduction by the

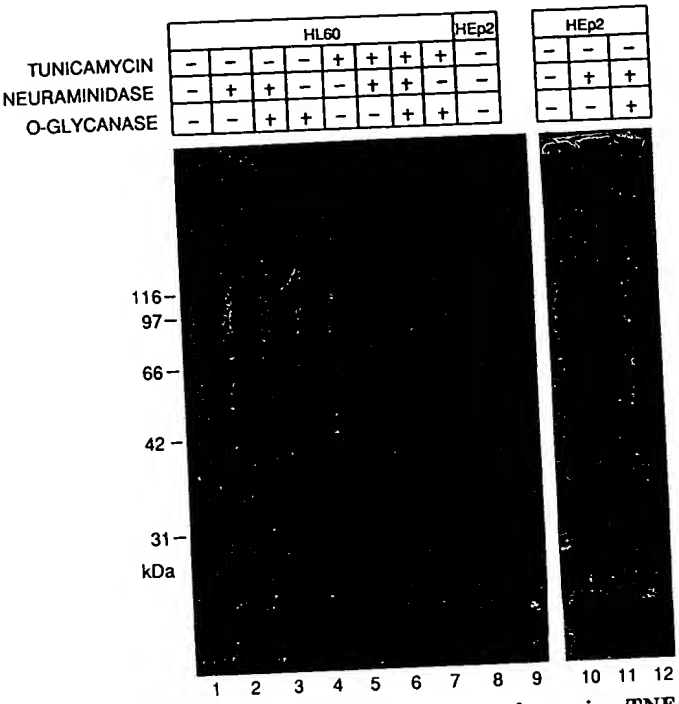


FIG. 9. **O-Linked sugars are present on the major TNFα receptor of HL60 cells but were not detected on the TNFα receptors of HEp2 cells.** Cells grown in the absence or presence of tunicamycin were incubated with radiolabeled TNFα and cross-linker, were lysed by sonication, and 1500 × *g* supernatants were prepared. These extracts were then incubated with the indicated combination of deglycosylation enzymes and subsequently analyzed by SDS-PAGE and autoradiography.

O-glycanase (Fig. 9, *lanes 4*, *5*, *7*, and *8*).

A slight reduction of 2–4 kDa was seen upon neuraminidase treatment of the 75-kDa cross-linked product of HEp2 cells (Fig. 9, *lanes 10* and *11*). However, no evidence for an additional down-shift by *O*-glycanase was obtained (Fig. 9, *lanes 11* and *12*), indicating that the sialic acid residues are part of the *N*-linked sugars and *O*-linked sugars might be absent.

Thus, the 100-kDa cross-linked product of HL60 cells contains both *N*- and *O*-linked carbohydrate chains, which in total contribute 10–15 kDa to its molecular mass. Again, the major TNFα receptors from HL60 cells and HEp2 cells behaved differently.

DISCUSSION

Most cells contain high affinity cell surface receptors for TNFα, and these receptors are believed to mediate at least some of the cellular effects of TNFα. How many different types of TNFα receptors are existing? Answering this question and especially the question whether different receptors have different biological characteristics may be very important to determine why cells respond differently to exposure to TNFα. Cross-linking studies were performed to visualize TNFα receptors. Different molecular masses have been reported for cross-linked complexes between radiolabeled TNFα and cell surface receptors (see Introduction and below). It remained, however, unclear what the relationship was between these different cross-linked products.

In this paper, we describe a biochemical characterization of the major TNFα receptors found on myeloid cells (HL60, U937, K562, and freshly isolated blood monocytes) and on cells of epithelial origin (MCF7, HEp2, and HeLa cells). We show that the major surface receptors for TNFα on these different cell types are different proteins. The receptors were visualized by cross-linking radiolabeled TNFα to the cell surface receptor(s) under mild cross-linking conditions, under which only a small fraction of the labeled TNFα molecules, bound to the cell, was cross-linked to the receptor. The pattern of cross-linking shown in this paper remained similar over a wide range of cross-linker concentrations, except for high molecular weight aggregates that did not enter the gels (not shown). We assumed that the amount of the cross-linked products are representative of the actual amount of a TNFα receptor on these cells. This seemed likely since the amount of the major cross-linked TNFα-receptor complexes correlated well, over a wide range of TNFα concentrations, with the total amount of TNFα bound to the cells; and the cross-linking patterns were not different if other cross-linkers with different molecular spans were used (not shown).

Both the TNFα binding sites of myeloid cells and of the cells of epithelial origin were of high affinity (K_d values of 7.14×10^{-11} for HL60 cells and 3.26×10^{-10} for HEp2 cells). However, the cross-linked patterns were drastically different. Myeloid cells had major cross-linked products of 98–100 kDa, although minor bands were also observed, even if low concentrations of radiolabeled TNFα were used. The epithelial cells used here had a major cross-linked product of 75 kDa, a less abundant product of 95 kDa, and some additional minor bands. This pattern may not be characteristic for all epithelial or adherent cells, because the colon carcinoma cell line SW480, which may be of epithelial origin, has the 100-kDa product as the major cross-linked product (not shown). Our results for the myeloid cells agreed well with the 100–102-kDa cross-linked products reported by others for U937 cells and monocytes (26, 28) but are at variance with 92- and 75-kDa cross-linked products reported for U937 cells by Israel *et al.* (25). We also observed some minor cross-linked products of lower apparent molecular masses in myeloid cells. The ratio between the 100-kDa product and these minor products in HL60 cells was somewhat variable from experiment to experiment. However, the major cross-linked products of myeloid cells always were 98–100 kDa. Cross-linked products of 95 (92) and 75 kDa, as we observed in the epithelial cells, were also reported by others (18), while even four different cross-linked products were found in MCF7 cells (138, 90, 75, and 54 kDa) (27). Of these, the 138-kDa product was apparently only found in TNF-sensitive variants of MCF7.

Our biochemical and immunological characterization of TNFα receptors showed the following differences between the major 100-kDa cross-linked product of HL60 cells and the 75 kDa (and 95 kDa) cross-linked products of HEp2 cells: 1) the apparent molecular masses of the cross-linked products were different, regardless of the presence or absence of *N*- and *O*-linked glycosylation (see below); 2) no evidence was obtained for specific proteases (intracellular, cell surface located or secreted proteases), produced by HEp2 cells but not by HL60 cells, which could convert the major HL60 receptor into a protein similar to the major receptors of HEp2 cells; 3) the cross-linked products were differently glycosylated (see below); 4) the native TNFα-receptor complexes of both cell types showed different proteolytic fingerprints; 5) the tryptic fragments differed in their association with the cell membrane vesicles; 6) high but slightly different affinities for TNFα were calculated by Scatchard analysis; and 7) the 75- and 95-kDa complexes could be immunoprecipitated with antibodies against a TNFα receptor protein, whereas the 100-kDa complex could not be immunoprecipitated. The immunological differences between these receptor proteins were also confirmed by detection of the noncross-linked TNFα receptors of HL60 and HEp2 cells after SDS-PAGE by incubation of Western blots with either radiolabeled TNFα or an anti-

TNFα receptor antibody. HL60 cells contained an additional protein that binds TNFα but not the anti-receptor antibody.[2] These data suggest that the major receptor proteins of HL60 and HEp2 cells, as visualized by cross-linking to radiolabeled TNFα, are different proteins. Of course, we cannot yet determine whether these proteins are encoded by different genes or represent different products derived from one single TNFα receptor gene.

The 100-kDa major cross-linked product of HL60 cells contained *N*- and *O*-linked glycosylation, in total contributing about 10–15 kDa to the receptor's molecular mass. The *N*-linked glycosylation contributed 2–4 kDa, whereas *O*-linked glycosylation contributed an additional 6–10 kDa. The cross-linked products of different myeloid cells did not co-migrate. However, the cross-linked products obtained if tunicamycin-grown cells were used had similar molecular masses, suggesting that the slightly different molecular masses of the cross-linked products of different myeloid cells may be result of differences in their *N*-linked carbohydrate chains.

The major 75 kDa and minor 95 kDa products of HEp2 cells also contained *N*-linked sugars, including sialic acid residues, but may lack *O*-linked sugars. The total amount of cross-linked complexes per cell was, however, drastically reduced if tunicamycin-grown cells were used, suggesting that the final sizes of the deglycosylated complexes might be the combined result of removal of the *N*-linked sugars and proteolytic digestion.

For the myeloid cells HL60 and U937, the *N*-linked glycosylation was found on a proteolytic fragment, which represented (part of) the receptor's extracellular domain. This trypsin fragment containing the bound TNFα did not pellet together with the membranes. For HEp2 and MCF7 cells, the trypsin fragments containing TNFα and the *N*-linked sugars pelleted together with the membrane fragments, which may mean that they still contain a transmembrane region. The *N*-linked glycosylation was not essential for the binding of TNFα to the major receptor of myeloid cells. The number of TNFα binding sites per HL60 cell was reduced about 50% in the tunicamycin-grown cells. This value correlated well with the 50% reduction in the rate of protein synthesis in these tunicamycin-grown cells (not shown).

We suggest that the myeloid cells have a major receptor that is present in the 98–100-kDa cross-linked complexes. All published evidence (34, 35) suggests that TNFα binds as a trimer to its receptor. However, since we use cross-linking conditions in which a large fraction of the TNFα molecules was not cross-linked, it seems reasonable to assume that the 98–100-kDa complexes are generated by a single cross-linking reaction involving one of the three TNFα molecules of the bound trimer and the receptor protein. This suggests a molecular mass of about 80 kDa for the fully glycosylated receptor and about 65–70 kDa for the receptor devoid of *N*- and *O*-linked glycosylation. This receptor protein does not react with our anti-receptor antibodies. The epithelial cells lack the 80-kDa TNFα receptor but contain at least one other type of TNFα receptor, which is present in the 75-kDa cross-linked product. This suggests a size of 55–60 kDa for the fully glycosylated second receptor. The receptors contained in the major 75- and minor 95-kDa complexes found with HEp2 cells might be very similar, because they can be precipitated with the same anti-receptor antibody. However, the exact relationship between the 75- and 95-kDa cross-linked products is at present unclear. In addition, the immunoprecipitation data with our anti-receptor antibodies suggest that HL60 cells also contain a small amount of immunoreactive receptor protein of undefined size. Previously, it was unclear whether these different major receptors found on myeloid and epithelial cells have the same or different functions. We are currently investigating this point.

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immunodominant region of the autoantigen MBP. This may provide insight into the molecular mechanisms of MS and help in the design of new specific therapeutic approaches.

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5. Sequences of MBP peptides: MBP(84–102): DENPVVHFFKNIVTPRTPP MBP(143–168): FKGVDAQGTLSKIFKLGGRD. Single-letter abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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11. Myelin basic protein–specific T cell lines were grown from peripheral blood mononuclear cells at 200,000 cells per well in the presence of human MBP (10 μg/ml). Under these conditions 1 to 20% of the wells were positive for MBP; therefore, most lines are likely to have been generated from a single MBP-reactive T cell. Cells were stimulated two times with MBP and tested for their peptide specificity by use of a panel of 13 overlapping synthetic MBP peptides. All cell lines analyzed reacted specifically with one of the 13 synthetic MBP peptides (*4*). After a third stimulation with the specific MBP peptide, RNA was extracted from cell culture pellets (20,000 to 50,000 cells) by extraction with guanidinium isothiocyanate/phenol chloroform and isopropranol precipitation in the presence of carrier tRNA. Single-stranded cDNAs were synthesized with oligo-dT and avian myeloblastosis virus reverse transcriptase. PCR amplification was done with a panel of 19 oligonucleotides corresponding to the CDR2 region of the TCR β chain and a C_β primer. Amplifications were done for 30 cycles (94°C, 1 min; 55°C, 2 min; and 72°C, 3 min) with 1 μg of each primer in 50-μl reactions. Amplified products were separated in 1% agarose gels, transferred to nitrocellulose, and hybridized with an internal oligonucleotide probe. Probes were end-labeled with [γ-^{32}P]ATP (adenosine triphosphate) and T4 polynucleotide kinase to a specific activity of 10^8 cpm/μg and hybridized. Blots were washed at a final stringency of 6× SSC (saline sodium citrate) at 70°C and autoradiographed for 2 to 18 hours. T cell lines that were positive for more than two V_β segments were considered not to be derived from a single MBP-reactive T cell and were therefore excluded from analysis. For sequencing, amplification was performed with a V_β17 primer specific for the leader segment, which contained an internal Pst I restriction site. Amplified DNA was treated with proteinase K, extracted with phenol chloroform, precipitated with ethanol, and digested with restriction endonucleases Bgl II and Pst I. Gel-purified DNA was ligated into M13mp19, and single-stranded DNA was sequenced by the dideoxy method. Negative controls were included during the procedure to test for possible contamination of RNA samples or reagents used for cDNA synthesis and amplification.
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14. Primers used for PCR: V_β1, 5′ AAGAGAGAGCAAAAGGAAACATTCTTGAAC 3′; V_β2, 5′ GCTCCAAGGCCACATACGAGCAAGGCGTCG 3′; V_β3, 5′ AAAATGAAAGAAAAAGGAGATATTCCTGAG 3′; V_β4, 5′ CTGAGGCCACATATGAGAGTGGATTTGTCA 3′; V_β5, 5′ CAGAGAAACAAAGGAAACTTCCCTGGTCGA 3′; V_β6, 5′ GGGTGCGGCAGATGACTCAGGGCTGCCCAA 3′; V_β7, 5′ ATAAATGAAAGTGTGCCAAGTCGCTTCTCA 3′; V_β8, 5′ AACGTTCCGATAGATGATTCAGGGATGCCC 3′; V_β9, 5′ CATTATAAATGAAACAGTTCCAAATCGCTT 3′; V_β10, 5′ CTTATTCAGAAAGCAGAAATAATCAATGAG 3′; V_β11, 5′ TCCACAGAGAAGGGAGATCTTTCCTCTGAG 3′; V_β12, 5′ GATACTGACAAAGGAGAAGTCTCAGATGGC 3′; V_β14, 5′ GTGACTGATAAGGGAGATGTTCCTGAAGGG 3′; V_β15, 5′ GATATAAACAAAGGAGAGATCTCTGATGGA 3′; V_β16, 5′ CATGATAATCTTTATCGACGTGTTATGGGA 3′; V_β17, 5′ TTTCAGAAAGGAGATATAGCTGAAGGGTAC 3′; V_β18, 5′ GATGAGTCAGGAATGCCAAAGGAACGATTT 3′; V_β19, 5′ CAAGAAACGGAGATGCACAAGAAGCGATTC 3′; V_β20, 5′ ACCGACAGGCTGCAGGCAGGGGCCTCCAGC 3′; C_β, 5′ GGCAGACAGGACCCCTTGCTGGTAGGACAC 3′; C probe, 5′ TTCTGATGGCTCAAACACAGCGACCTCGGG 3′; V_β17 leader, 5′ AGCAACCAGGTGCTCTGCAGTGTGGTCCTT 3′; and J_β2.1, 5′ CCCTGGCCCGAAGAACTGCTCATTGTAGGA 3′.
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A Receptor for Tumor Necrosis Factor Defines an Unusual Family of Cellular and Viral Proteins

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Tumor necrosis factor α and β (TNF-α and TNF-β) bind surface receptors on a variety of cell types to mediate a wide range of immunological responses, inflammatory reactions, and anti-tumor effects. A cDNA clone encoding an integral membrane protein of 461 amino acids was isolated from a human lung fibroblast library by direct expression screening with radiolabeled TNF-α. The encoded receptor was also able to bind TNF-β. The predicted cysteine-rich extracellular domain has extensive sequence similarity with five proteins, including nerve growth factor receptor and a transcriptionally active open reading frame from Shope fibroma virus, and thus defines a family of receptors.

TUMOR NECROSIS FACTOR α (TNF-α, cachectin) and β (TNF-β, lymphotoxin) are structurally and functionally homologous proteins secreted by activated macrophages and lymphocytes, respectively (*1*). These cytokines have pleiotropic activities in vitro and in vivo, including cytotoxic effects against tumors and virus-infected cells, stimulation of interleukin-1 secretion, stimulation of prostaglandin E2 and collagen production, inhibition of lipogenic gene expression in adipocytes, and stimulation of various immune effector cells (*2*). Clinical interest has focused on TNF because it appears to be a common mediator of inflammation, endotoxin-induced shock (*1*), and the wasting syndrome commonly observed in chronic infections and neoplastic disease (*3*). TNF receptors appear on virtually all somatic cells (*1*), and generally the ligands cross-compete for binding (*4*), suggesting they share a common receptor. As an aid to studying the TNF system in molecular detail, we isolated a cDNA clone of the receptor.

The SV40-transformed human lung fibroblast cell line WI26-VA4 was used as a source of mRNA for construction of a cDNA library. This cell line binds both TNF-α and -β and displays multiple affinity classes; approximately 23,000 binding sites per cell (N) were detected with ^{125}I–TNF-α that could be fit to two affinity classes, low (K_{a1} = 0.16 ± 0.10 nM^{-1}, N_1 = 19,700 ±

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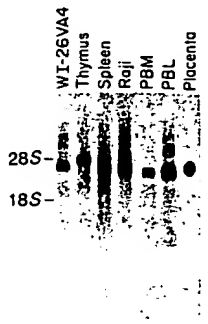
4,800) and high (K_{a2} = 6.2 ± 3.9 nM^{-1}, N_2 = 3,000 ± 1,400) (Fig. 1A). TNF-β binds with lower affinity than TNF-α and the ligands cross-compete for binding (Fig. 1B). Double-stranded cDNA was synthesized by standard procedures, inserted into the mammalian expression vector pDC302 (*5*) and a TNF receptor clone isolated by a direct expression approach. Plasmid DNA from about 1000 *Escherichia coli* (DH5α) transformants were pooled, transfected into COS cells, and screened by contact autoradiography (*6*), which detects positive pools by the ability of those COS cells expressing TNF receptor inserts to bind ^{125}I-labeled TNF-α. After screening 175,000 clones, one positive pool (#737) was obtained, subdivided, and converged to a single clone in two cycles of this procedure. By autoradiographic plate binding (*6*), the pure clone when transfected into COS cells expressed a receptor that bound both ^{125}I–TNF-α and -β; binding of either ligand was completely inhibited by a 200-fold excess of the same or homologous unlabeled cytokine (*7*). Quantitative in situ binding studies of the COS-expressed receptor with ^{125}I–TNF-α agreed with these results and showed the binding to be complex (Fig. 1C). As with the native WI26-VA4 receptor, the recombinant COS receptor displayed both low (K_{a1} = 0.18 ± 0.06 nM^{-1}) and high (K_{a2} = 10.1 ± 1.0 nM^{-1}) affinity classes for ^{125}I–TNF-α. TNF-β bound with lower affinity and competitively inhibited ^{125}I–TNF-α binding (Fig. 1D). Thus, ligand binding properties of both the native and recombinant receptor appear similar. The origin of the multiple affinity classes for TNF-α is unclear. Indeed, most workers (*1, 4, 8, 9*), but not all (*10*), have reported monophasic Scatchard plots for TNF-α. However, TNF-α is predominantly a homotrimer (*11*) and therefore intrinsically capable of multivalent binding. In one report (*12*), differential biological effects could be related to biphasic binding of TNF-α. While not necessarily sharing a common origin, multiple affinity classes are a common feature of many receptor systems (*13*).

The isolated TNF receptor cDNA was used as a probe to analyze the mRNA expressed in a variety of cell lines and tissues (Fig. 2). A single size class of transcripts of ~4.5 kb was detected in WI26-VA4, Raji cells (a B lymphoblastoid line), LPS-stimulated peripheral blood monocytes (PBM), induced peripheral blood T cells (PBL), and placental tissue. A transcript of slightly larger size (~5.0 kb) was detected in thymic tissue, and splenic tissue contained transcripts of both size classes. The origin of these differences is not clear, but the presence of TNF receptor transcripts in these different cells is consistent with the near ubiquitous distribution of the receptor.



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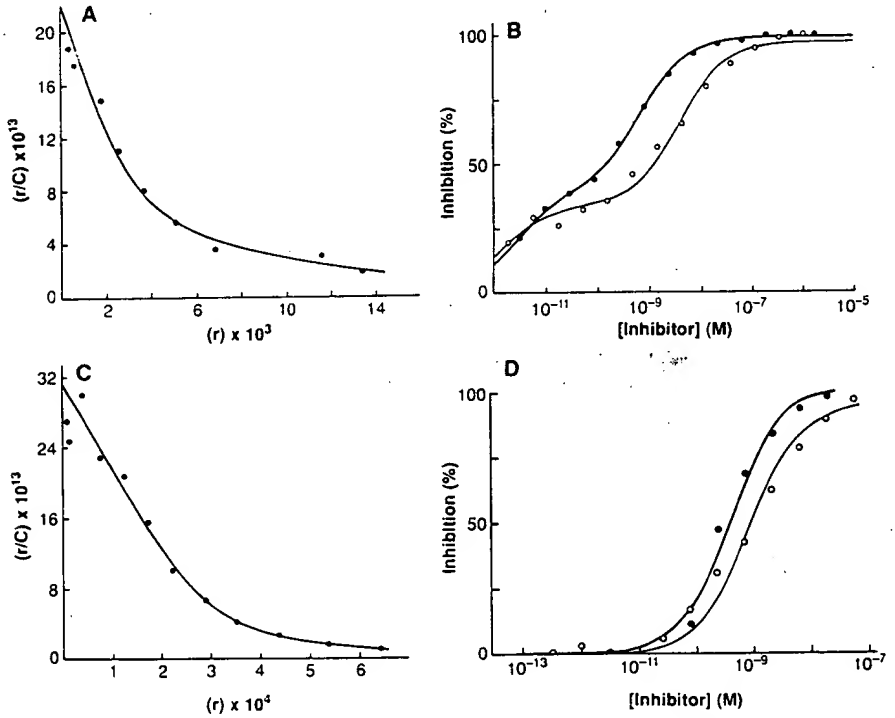

Fig. 1. TNF binding characteristics of native and recombinant TNF receptors (*31*). (**A**) Direct binding of ^{125}I–TNF-α to WI26-VA4 cells (Scatchard coordinate system). (**B**) Inhibition of ^{125}I–TNF-α binding to WI26-VA4 cells by unlabeled TNF-α (●) and TNF-β (○). TNF-α inhibition: $K_{I,1}$ (low affinity) = 1.6 ± 0.2 nM; $K_{I,2}$ (high affinity) = 0.8 ± 0.1 pM. TNF-β inhibition: $K_{I,1}$ (low affinity) = 0.29 ± 0.06 nM; $K_{I,2}$ (high affinity) = 1.3 ± 0.6 pM. (**C**) Direct binding of ^{125}I–TNF-α to recombinant (COS) TNF receptor. (**D**) High affinity site inhibition of ^{125}I–TNF-α binding to recombinant (COS) TNF receptor by unlabeled TNF-α (●) or -β (○). K_I (α) = 6.7 ± 2.9 nM; K_I (β) = 3.3 ± 0.8 nM. C, free concentration of TNF (molar); r, molecules of TNF bound per cell. All parameter values are ± standard error. Data fit to one or two site models as described (*32*).

4,800) and high (K_{a2} = 6.2 ± 3.9 nM^{-1}, N_2 = 3,000 ± 1,400) (Fig. 1A). TNF-β binds with lower affinity than TNF-α and the ligands cross-compete for binding (Fig. 1B). Double-stranded cDNA was synthesized by standard procedures, inserted into the mammalian expression vector pDC302 (*5*) and a TNF receptor clone isolated by a direct expression approach. Plasmid DNA from about 1000 *Escherichia coli* (DH5α) transformants were pooled, transfected into COS cells, and screened by contact autoradiography (*6*), which detects positive pools by the ability of those COS cells expressing TNF receptor inserts to bind ^{125}I-labeled TNF-α. After screening 175,000 clones, one positive pool (#737) was obtained, subdivided, and converged to a single clone in two cycles of this procedure. By autoradiographic plate binding (*6*), the pure clone when transfected into COS cells expressed a receptor that bound both ^{125}I–TNF-α and -β; binding of either ligand was completely inhibited by a 200-fold excess of the same or homologous unlabeled cytokine (7). Quantitative in situ binding studies of the COS-expressed receptor with ^{125}I–TNF-α agreed with these results and showed the binding to be complex (Fig. 1C). As with the native WI26-VA4 receptor, the recombinant COS receptor displayed both low (K_{a1} = 0.18 ± 0.06 nM^{-1}) and high (K_{a2} = 10.1 ± 1.0 nM^{-1}) affinity classes for ^{125}I–TNF-α. TNF-β bound with lower affinity and competitively inhibited ^{125}I–TNF-α binding (Fig. 1D). Thus, ligand binding properties of both the native and recombinant receptor appear similar. The origin of the multiple affinity classes for TNF-α is unclear. Indeed, most workers (*1, 4, 8, 9*), but not all (*10*), have reported monophasic Scatchard plots for TNF-α. However, TNF-α is predominantly a homotrimer (*11*) and therefore intrinsically capable of multivalent binding. In one report (*12*), differential biological effects could be related to biphasic binding of TNF-α. While not necessarily sharing a common origin, multiple affinity classes are a common feature of many receptor systems (*13*).

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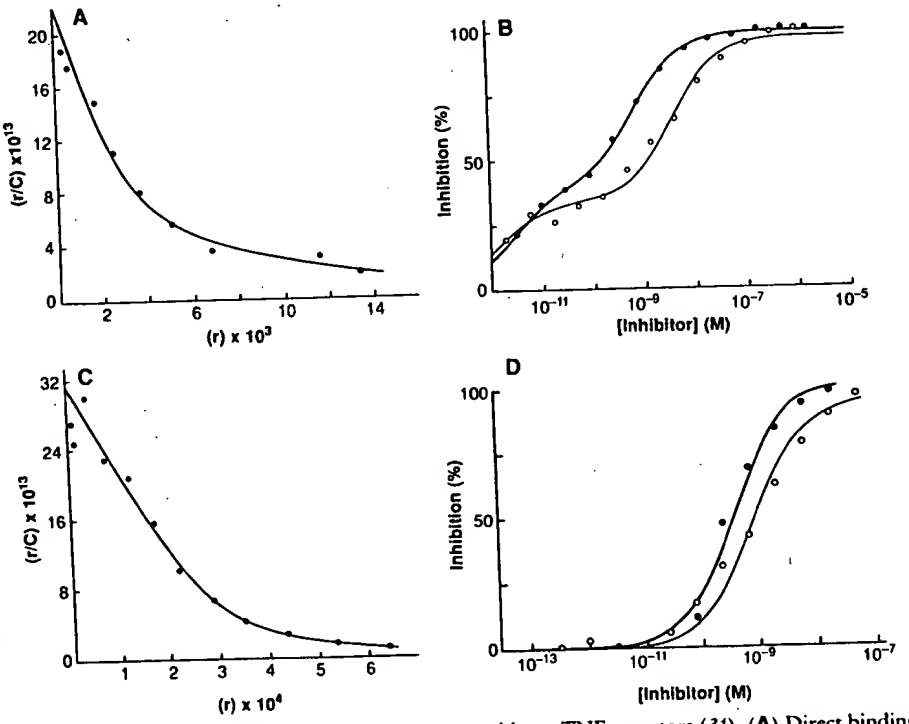

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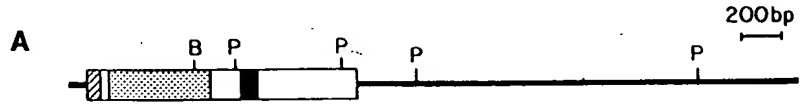
cysteines (22 residues) and also contain two potential N-linked glycosylation sites. The receptor terminates in a cytoplasmic domain of 174 amino acids, rich in serines (18%), six of which are contiguous. Five cysteines and one potential N-linked glycosylation site are also present in this domain.

A computer search of several sequence databases (*19*) queried with the entire 439-residue sequence of the mature TNF receptor revealed five proteins with striking similarity: human and rat NGF receptor, CD40, cDNA clone 4-1BB, and T2 (Fig. 4). Four of these are transmembrane proteins, two of which are known receptors (for human and rat NGF). CD40 is a B cell–localized surface antigen, found also on neoplastic cells of epithelial origin, that becomes phosphorylated in the cytoplasmic domain after binding the CD40-specific monoclonal antibody G28-5 (*20*). Clone 4-1BB was identified as a murine cDNA from induced helper and cytolytic T cell clones (*21*). Both molecules have been suggested to be cytokine receptors for unidentified ligands. All identity between these four proteins is localized to the cysteine-rich regions of the extracellular domains; no homology was detected between the TNF receptor cytoplasmic domain and any proteins in the database. T2 is a transcriptionally active open reading frame from the Shope fibroma virus (SFV), a poxvirus that produces invasive malignancies in newborn rabbits (*22*). Although dominated by 22 conserved cysteines, the alignment is also reinforced by other conserved amino acids, particularly tyrosine, glycine, and proline. Thus, the extracellular domains of these molecules, presumably heavily disulfide bonded, probably share a common structural motif. Central to this motif would appear to be repeating homologous domains. Several groups have shown that the cysteine-rich regions of NGF receptor and CD40 can be resolved into either pseudo twofold repeats of about 80 amino acids or pseudo fourfold repeats of about 40 residues (*17, 20*). Similar repeats can be shown with the TNF receptor and T2, consistent with all these genes having arisen by duplication and divergence from a common gene. Since both NGF and TNF are oligomeric, repeating substructures in their receptors may aid in binding and predicts that the putative ligands for CD40 and 4-1BB may also be oligomers. The net charge associated with the cysteine-rich domains of these family members varies (−19 for NGF receptor; +1 for TNF receptor), which may be related to ligand specificity. Presumably, it is this NH_2-terminal region that contains the TNF binding site. Multiple lines of evidence have localized the (apoprotein B) ligand binding site of the LDL receptor to the NH_2-terminal (60-kD), cysteine-rich



B

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  1  MAPVAVWAAL AVGLELWAAA HALPAQVAFT PYAPEPGSTC RLREYYDQTA
 51  QMCCSKCSPG QHAKVFCTKT SDTVCDSCED STYTQLWNWV PECLSCGSRC
101  SSDQVETQAC TREQNRICTC RPGWYCALSK QEGCRLCAPL RKCRPGFGVA
151  RPGTETSDVV CKPCAPGTFS NTTSSTDICR PHQICNVVAI PGNASMDAVC
201  TSTSPTRSMA PGAVHLPQPV STRSQHTQPT PEPSTAPSTS FLLPMGPSPP
251  AEGSTGDFAL PVGLIVGVTA LGLLIIGVVN CVIMTQVKKK PLCLQREAKV
301  PHLPADKARG TQGPEQQHLL ITAPSSSSSS LESSASALDR RAPTRNQPQA
351  PGVEASGAGE ARASTGSSDS SPGGHGTQVN VTCIVNVCSS SDHSSQCSSQ
401  ASSTMGDTDS SPSESPKDEQ VPFSKEECAF RSQLETPETL LGSTEEKPLP
451  LGVPDAGMKP S
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Fig. 3. Sequence of the human TNF receptor cDNA clone. (**A**) Schematic representation and restriction map of the cDNA. The entire coding region is boxed. The leader is hatched, the cysteine-rich region is shown stippled, and the transmembrane segment is solid. B = Bgl II; P = Pvu II. (**B**) The deduced amino acid sequence of cDNA coding region. The leader region is singly underlined, the transmembrane domain is shown boxed, potential N-linked glycosylation sites are doubly underlined, and cysteines are identified by an asterisk. The entire nucleotide sequence is available upon request and has been deposited at GenBank, accession number M32315.

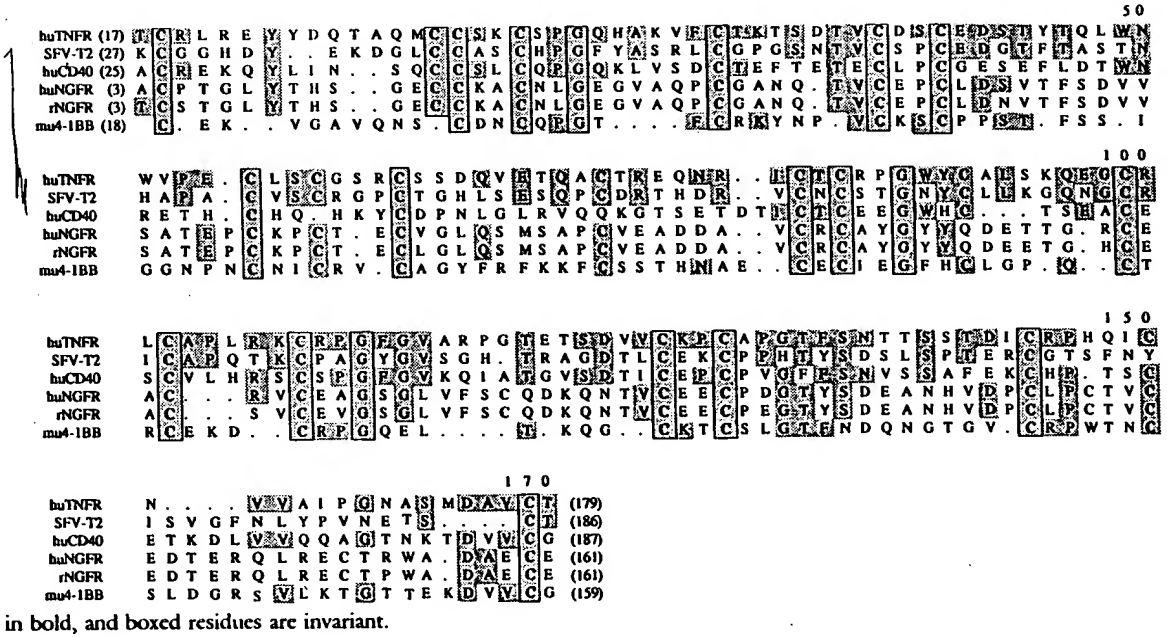

Fig. 4. Sequence similarities among the TNF receptor superfamily. Consensus alignment of residues from the cysteine-rich regions of human TNF receptor (huTNFR), T2 open reading frame of Shope fibroma virus (SFV-T2), human CD40 (huCD40), human and rat nerve growth factor receptor (huNGFR and rNGFR), and murine cDNA clone 4-1BB (mu4-1BB). Numbers at NH_2- and COOH-termini refer to residues as cited in publications describing cDNA cloning (*17, 20, 21, 22*); numbers at top right of each block mark residues from NH_2-terminus at top left. Shaded residues reflect those common to huTNF receptor and at least one other protein. Cysteines are in bold, and boxed residues are invariant.

domain (*18*).

Sequences containing cysteine-rich repeats are present in a number of proteins, including the CD18 adhesion molecules (*23*), epidermal growth factor (EGF) precursor, *Drosophila notch* protein, the *neu* oncogene, and the external domains of receptors for LDL, EGF, and insulin (*18, 24*). Although many of these proteins show homology to each other, we detect little similarity to the TNF receptor. Optimal alignments of family members using the National Biomedical Research Foundation (NBRF) ALIGN program (*19*) show the strongest similarity is between the TNF receptor and T2, with a score of 19 standard deviations (SD) above the mean score for an ensemble of randomly permuted molecules of the same lengths and amino acid composition. ALIGN scores greater than 3.0 are considered significant and indicate common ancestry. Almost 40% of the residues are identical, approaching the conservation level between many murine and human cytokines and their receptors (*25*). Slight variants of T2 may also exist in other poxvirus family members, and some of these viruses are strongly immunosuppressive (*22*). Although T2 possesses a signal peptide sequence, the molecule appears to lack a hydrophobic segment typical of transmembrane regions, suggesting that T2 may be a soluble entity secreted from virally infected cells. Thus, perhaps T2 may bind TNF, or another cytokine, serving to locally dampen the host immune response. The protective effects of such a "soluble receptor" would no doubt confer a selective advantage to the pathogen. CD40, however, is also similar to this TNF receptor (38.5% amino acid identity; 15.2 SD), yet does not bind TNF-α when expressed in COS cells at high levels in an immunoreactive form (*26*). TNF receptor is more distantly related to 4-1BB and NGF receptor (9.0 and 12.3 SD, respectively).

The signal transduction mechanism of TNF is unclear. The receptor cytoplasmic domain, as with other family members, shows no similarity with known proteins, including the cytoplasmic domain of the human T cell interleukin-1 (IL-1) receptor (*6*), despite the fact that TNF and IL-1 mediate many common biological activities (*1*). The TNF receptor expressed in COS cells does not bind radiolabeled human IL-1α or -β, nor does the recombinant human IL-1 receptor bind TNF (*7*). No sequences present are typical of tyrosine kinases, protein kinase C, or phosphorylation sites corresponding to substrates for these kinases (*27*). The cytolytic activity of TNF, however, appears to depend on the presence of a 200-kD protein distinct from the receptor, and with which it comodulates (*28*).

Several groups have characterized TNF binding proteins from urine. Uromodulin is a renal glycoprotein that binds IL-1, IL-2, and TNF-α with high affinity, but does not inhibit ligand binding to their respective receptors and shows no sequence similarity to the TNF receptor reported here (*29*). Two groups have recently reported purification and sequencing of soluble TNF-α binding proteins from urine with molecular weights of 27 to 30 kD (*30*). However, the NH_2-terminal sequence of these proteins is not found in the predicted sequence of clone 737. TNF-α receptors on myeloid cells are probably different from those on cells of epithelial origin (*8*). An 80-kD form of the receptor contains O- and N-linked carbohydrate; a 60-kD form lacks O-linked carbohydrate, possesses a different form of N-linked carbohydrate, and displays different tryptic peptide maps. Monoclonal antibodies to these two receptors also do not cross-react. The receptor we have described may correspond to the 80-kD form. Affinity cross-linking of the recombinant receptor using either ^{125}I–TNF-α or -β shows a single species of 80 kD (*7*). Because the calculated protein is 46 kD, carbohydrate appears to be attached, and both O- and N-linked glycosylation sites are present in the sequence.

The availability of a full-length cDNA clone for a human TNF receptor will now permit detailed studies into the molecular mechanisms by which ligand-receptor interactions produce the pleiotropic effects of this important cytokine. Soluble, recombinant forms of this receptor may also be produced to explore the clinical value of TNF inhibition in pathological settings.

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26. The CD40 and human TNF receptor clones were separately transfected into COS cells (CD40-COS, TNFr-COS) and expression was monitored by radiolabeled ligand binding using contact autoradiography (*6*). CD40-COS clearly and specifically bound ^{125}I–G28 antibody, but did not bind ^{125}I–TNF-α (0.2 nM). ^{125}I-G28 binding was not inhibited by a 500-fold molar excess of unlabeled TNF-α. Similarly, TNFr-COS specifically bound ^{125}I–TNF-α and -β but not ^{125}I-G28 (0.2 nM).
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31. COS cells were transfected with the vector pDC302 containing the TNF receptor cDNA insert (clone 737) or control vector lacking insert as described (*5, 6*). For quantitative in situ binding studies, transfected COS cells were replated (24 hours after transfection) into six well trays (CoStar) and analyzed 48 hours later at near confluence (6×10^5 cells per well). COS monolayers were washed once with phosphate-buffered saline (PBS), then incubated with ^{125}I–TNF-α at various concentrations in binding media [RPMI 1640, bovine serum albumen (10%), NaN_3 (0.1%), 20 mM Hepes, pH 7.4] at 4°C for 2 hours. Free ^{125}I–TNF-α was determined by counting gamma emissions in the supernatant. Monolayers were then washed once with ice-cold RPMI, detached with 0.1% trypsin in PBS, and counted to determine bound ligand. Nonspecific ligand binding was determined by inclusion of a 200-fold molar excess of unlabeled ligand. Inhibition assays used ^{125}I–TNF-α at 0.2 nM. Data were analyzed and theoretical curves plotted as described (*6, 32*). TNF-α and TNF-β (R&D Sciences) were radiolabeled using Iodogen (Pierce) to a specific activity of 2×10^{15} cpm/mmol (*4*). Radiolabeled TNF-α gel filtered as a single peak with an apparent molecular weight of 55 kD (*7*), consistent with a trimeric status (*11*).
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"The good news is we have the human genome. The bad news is the computer alphabetized it."

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Biochemistry

Complementary DNA cloning of a receptor for tumor necrosis factor and demonstration of a shed form of the receptor

(tumor necrosis factor-binding protein/cachexia/inflammation)

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ABSTRACT **Tumor necrosis factor (TNF) receptor (TNFR) was isolated as a 68-kDa glycoprotein from UC/HeLa 2-5 cells developed from a parental B-cell line (UC cells) to overexpress the receptor. Tryptic digests of two separate TNFR preparations provided amino acid sequences of four different peptides. Amino-terminal analysis indicated the presence of the amino-acid sequence Val-Ala-Phe-Thr-Pro, reported to be the amino-terminal sequence of a 30-kDa urinary TNF-binding protein II. Examination of the cultured medium of UC/HeLa 2-5 cells showed an abundance of a 40-kDa TNF-binding protein, indicating that the previously cited 30-kDa TNF-binding protein II is likely to be a shed form of the TNFR. Based on the peptide sequences, oligonucleotides were synthesized, and two of these were used as primers in the polymerase chain reaction to amplify cDNA sequences from poly(A)$^+$ RNA of UC/HeLa 2-5 cells. These PCR fragments were radiolabeled and used to screen a cDNA library made from UC/HeLa 2-5 mRNA. Further analysis identified cDNA sequences that encoded the amino acid sequences of all four TNFR peptides. RNA blot-hybridization analysis of UC/HeLa 2-5 mRNA revealed a 3.8-kilobase transcript of the same size as the mRNA in the parental UC cells. Genomic Southern blots indicated the presence of a single gene in parental cells and a second, amplified gene in TNFR-overexpressing cells, suggesting amplification of the transfected gene as a possible mechanism for the increase in TNFR numbers in UC/HeLa 2-5 cells.**

Tumor necrosis factor α (TNF-α) mediates a variety of responses by binding to specific high-affinity cell-surface receptors (1). The biological activities attributed to TNF are cytotoxicity toward certain tumors and tumor cells (2, 3), inhibition of adipose-specific genes (4–6), cachexia (1, 7), inflammation (8–10), and the mediation of endotoxin shock (11). Effects of TNF appear also to be mediated by inducing release of other cytokines such as interleukins 1 and 6 (1, 12) and arachidonic acid metabolites (ref. 1; G.M.R., unpublished results), some of which stimulate proliferation of a variety of cells (1, 13, 14).

TNF receptors (TNFRs) exhibit affinity constants for TNF in the nanomolar range. Receptor numbers vary from 1000 to 10,000 per cell (13, 15–17), and molecular size estimates of 54–138 kDa (17–20) have been deduced from complexes of cross-linked radiolabeled ligand to receptor. Because the number of the ligand molecules in these complexes is uncertain, these estimates have been tentative. Based on (*i*) the sizes of these receptor–ligand complexes, (*ii*) the analyses of tryptic fragments generated from these complexes, and (*iii*) the apparent absence of immunological cross-reactivity between receptors from different cell types, the existence of two different receptors has been proposed (21). In addition, the isolation of two distinct TNF-binding proteins (both kDa in size) from human urine has been provided as a evidence for the existence of different types of recep These urinary proteins are believed to represent sol forms of different receptors (22–24).

We have developed B-cell lines (among them UC/H 2-5) that express 150,000–180,000 TNFRs per cell Receptor protein purified from these cells has a mole size of ≈68–70 kDa and, when covalently linked with T forms complexes primarily of 87 kDa and to a lesser e 104 kDa. These observations suggest that TNF assoc predominantly in the monomeric form with the receptor In this paper we report the cloning of the cDNA fo TNFR§ from these receptor-amplified cells. The cDNA was isolated by using radiolabeled probes generated b polymerase chain reaction (PCR) with oligonucleotide p ers corresponding to amino acid sequences of the pu receptor. Amino-terminal analysis of the purified rec protein indicates identity with one of the urinary T binding proteins (24).

METHODS

Protein Purification, Peptide Analysis, and Protein Seq ing. TNFR was purified from ≈2×10^{11} UC/HeLa 2-5 hu B cells (25). The protein was analyzed by NaDodSO$_4$/P (26), electroblotted to nitrocellulose membrane (27), stained with 0.1% amido black. The 68-kDa TNFR pr band was treated with polyvinylpyrrolidone (PVP-4 block additional protein binding and was treated with tr (28, 29). Peptide fragments released into the supernatant resolved by reversed-phase HPLC. Edman degradatic the selected fragments in a gas-phase protein seque revealed the amino acid sequences of the peptides. An terminal sequence was determined by procedures desc by Matsudaira (30).

Filter Binding Assay and Ligand Blot Assay. These as were developed to identify soluble TNFR and were ducted as described (25).

RNA Isolation and Blot-Hybridization (Northern) Anal Cellular RNA was prepared by the guanidinium isothio nate procedure (31). Poly(A)$^+$ RNA selected by chrom raphy on oligo(dT)-cellulose (32) was denatured with gly and dimethyl sulfoxide (33), fractionated by electropho in 0.8% agarose gels in 10 mM sodium phosphate buffer 7.0), and transferred to Nytran membrane (Schleich Schuell). Prehybridization and hybridization with ran hexamer primed [α-^{32}P]dCTP-labeled cDNA probe (34) carried out at 42°C in 50% formamide containing 5× S

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Abbreviations: TNF, tumor necrosis factor; TNFR, tumor nec factor receptor; PCR, polymerase chain reaction.

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§The sequence reported in this paper has been deposited i GenBank data base (accession no. M35857).

(1× = 0.18 M NaCl/10 mM sodium phosphate, pH 7.4/1 mM EDTA), 5× Denhardt's solution (1× = 0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone), and 100 μg of carrier salmon sperm DNA per ml (35). The filters were washed at 58°C twice in 1× SSPE/0.1% $NaDodSO_4$ and twice with 0.1× SSPE/0.1% $NaDodSO_4$ and autoradiographed.

cDNA Cloning. cDNA libraries in the mammalian expression vector pCDM8 (36) were constructed by Invitrogen (San Diego, CA) from double-stranded oligo(dT)-primed cDNA synthesized from UC and UC/HeLa 2-5 cell poly(A)$^+$ RNA (37). The UC/HeLa 2-5 cDNA library contained $>1.3 \times 10^6$ recombinants, and the UC library contained $>0.7 \times 10^6$ recombinants. The UC/HeLa 2-5 library was screened with radiolabeled PCR-generated probes. Additional screening with the 21- or 23-base degenerate oligonucleotides (A, B, or C; see below) or the 51-base oligonucleotide (sequence D) was carried out by end-labeling with [γ-^{32}P]ATP and polynucleotide kinase. Filters hybridized with PCR probes were washed according to conditions described above. When oligonucleotides were used as probes, conditions that use tetramethylammonium chloride were used (38).

Oligonucleotide Synthesis. All oligonucleotides were synthesized by using a solid-phase phosphoramidite method on a Biosearch 8700-DNA synthesizer and, after deprotection, were purified by PAGE in 7 M urea. From peptide G523 (see Fig. 1), the 21-base oligonucleotide sequence corresponding to amino-acids 2 through 8 and called A in the sense orientation is 5'-ACN-CAR-GGN-CCN-GAR-CAR-CAR-CA-3', in which N = A, C, G, or T and R = A or G. From peptide G522 an oligonucleotide C' in the antisense orientation corresponding to amino-acids 2 through 8 is 5'-NGT-YTC-NGG-NGT-YTC-NAR-YTG-3', in which N and R are as before and Y = T or C. Also from peptide G522, a second oligonucleotide B' in the antisense orientation to amino acids 11 through 17 is 5'-NGG-YTT-YTC-YTC-NGT-NSW-NCC, in which Y and N are as before and S = G or C and W = A or T. Peptide G519-derived 51-base oligonucleotide sequence (D) based on human preferred codon usage is 5'-GAC-CAG-CCC-CAG-GCC-CCC-GGC-GTG-GAG-GCC-TCC-GGC-GCC-GGC-GAG-GCC-CGC-3'.

DNA Amplification. cDNA was prepared from poly(A)$^+$ RNA of UC/HeLa 2-5 cells and used as template (39). PCR-amplified specific DNA fragments were generated with the Perkin–Elmer/Cetus DNA thermal cycler according to conditions specified by the manufacturer. Briefly, 100 μl of the reaction mix contained 10 μl of 10× reaction buffer [670 mM Tris·HCl, pH 8.8/67 mM $MgCl_2$/1.7 mg of bovine serum albumin per ml/166 mM $(NH_4)_2SO_4$], 16 μl of each dNTP at 1.25 mM, 5 μl each of 20 μM primers, 10 μl of cDNA template (≈100 ng), 53.5 μl of H_2O, and 0.5 μl of *Thermus aquaticus* (*Taq*) polymerase (2.5 units per assay). Samples overlayed with 100 μl of mineral oil were subjected to 25 cycles of the following protocol: denaturation for 1 min at 95°C, annealing for 2 min at 55°C, elongation for 3 min at 72°C, and a final incubation for 7 min at 72°C. By using [^{32}P]dCTP and a mixture of oligonucleotides minus dCTP (40), high-specific-activity radiolabeled PCR probes were generated.

Southern Blot Analysis of cDNA Derived from pCDM8 Libraries and Genomic DNA from UC and UC/HeLa 2-5 Cells. cDNA inserts from the libraries were excised with *Xho* I or *Xba* I. Genomic DNA was isolated (35) and digested with different restriction enzymes, electrophoresed through 0.8% agarose in 89 mM Tris borate/2 mM EDTA, and transferred to Nytran membrane. Radiolabeled probes used are described in the figure legends. Hybridization and wash conditions were as described above for Northern blots.

DNA Sequence Analysis. The cDNA inserts from the selected pCDM8 clones were subcloned into M13mp19 (41) or pGemblue (Promega Biotec). The DNA was sequenced by the dideoxy chain-termination method using Sequenase (United States Biochemical) with ^{35}S-substituted deoxyadenosine 5'-[α-(^{35}S)thio] triphosphate (42).

RESULTS

TNFR Protein Analysis. Purified TNFR preparations contained a major polypeptide band of 68 kDa (Fig. 1*A*), which was judged to be the receptor by its high TNF-binding activity (25). From two separate purifications, 8 and 10 μg of the 68-kDa protein were subjected separately to trypsin treatment, and the released peptide fragments revealed the amino acid sequences shown in Fig. 1*B*. However, the amino acid sequence of the amino terminus was heterogeneous. The following residues were identified in the first five cycles of Edman degradation: (Ser or Val)-(Thr, Ala, or Pro)-(His, Pro, or Phe)-(Thr or Tyr)-(Ala, Pro, or Val); these residues are compatible with the sequence Val-Ala-Phe-Thr-Pro, reported to be the amino terminus of the urinary TNF-binding protein II (TNFBPII in ref. 24).

Presence of TNF-Binding Protein in the Culture Medium of Receptor-Amplified Cells. The heterogeneity in the amino-terminal sequence of the purified receptor plus the close identity of this sequence with the reported amino-terminal sequence of TNF-binding protein II (24) led us to analyze the growth medium of the cells for the presence of soluble forms of the receptor. The presence of TNF-binding protein was tested with the filter binding assay (25). Supernatants from suspension cultures of cells were slot-blotted to nitrocellulose membranes (Fig. 2*A*), incubated with ^{125}I-labeled TNF, washed, and autoradiographed (25). TNF-binding proteins were easily observed in the culture medium of UC/HeLa 2-5 cells but not of the parental UC cells. To estimate the size, TNF-binding proteins were enriched from the cultured medium by a TNF-Sepharose column, and the eluted fraction was used for a ligand blot assay (Fig. 2*B*). A major band of ≈40 kDa and a minor band of ≈30 kDa were identified.

Selection of Oligonucleotides as Primers and the Generation of Radiolabeled DNA Probes by PCR. Oligonucleotides A, B,

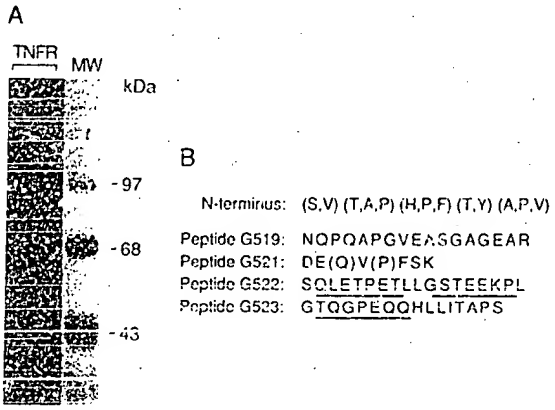

FIG. 1. (*A*) Amido-black stain of a purified preparation of TNFR from ≈2 × 10^{11} UC/HeLa 2-5 cells (25). The final protein fraction resolved by $NaDodSO_4$/PAGE in 10% acrylamide gels was electroblotted to nitrocellulose and stained with amido black. The arrow indicates the position of the TNFR polypeptide of 68 kDa. Tryptic fragments plus their amino acid sequences were obtained from this band. Lane MW shows molecular mass standards in kDa. (*B*) Amino acid sequences obtained are shown in single-letter code beginning at the amino terminus of each peptide. Underlined sequences in peptide G522 are the amino acid sequences against which oligonucleotides C and B were synthesized, respectively, while peptide G523 contains the amino acid sequence for oligonucleotide A. These oligonucleotides were used as primers in the PCR. A nucleotide sequence of 51 bases derived from peptide G519 (oligonucleotide D) was used as an oligonucleotide probe.

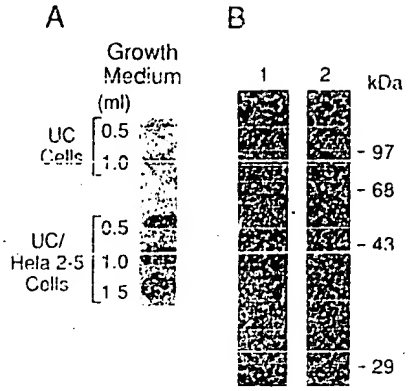

FIG. 2. (*A*) Filter binding assay to identify TNF-binding proteins in cultured media of UC and UC/HeLa 2-5 cells. Cells at a density of 1×10^6 per ml in RPMI 1640 medium containing 10% fetal bovine serum were centrifuged for 15 min at $1000 \times g$, and aliquots of the supernatant were slot-blotted to nitrocellulose membrane to immobilize TNFR. The filter was incubated with ^{125}I-labeled TNF to estimate relative amounts of TNFR as described (25). (*B*) Ligand blot analysis of purified TNFR and TNF-binding proteins shed into the growth medium. Growth medium from UC/HeLa 2-5 cells at a density of 1×10^6 cells per ml was collected and passed through a 3-ml bed volume of a TNF-Sepharose column. The column was washed, and the bound protein was eluted and subjected to ligand blot analysis for TNFR activity as described (25). Lane 1 contains the 68-kDa TNFR from UC/HeLa 2-5 cells as a control. Lane 2 contains the TNF-binding protein recovered from the growth medium of UC/HeLa 2-5 cells and shows a major band at 40 kDa.

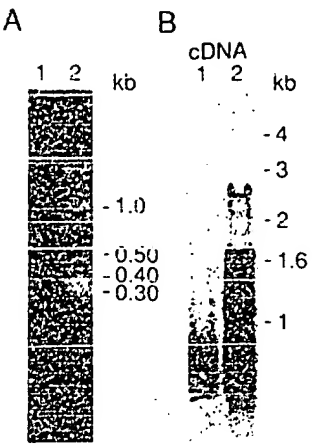

FIG. 3. (*A*) Gel electrophoresis of PCR-amplified DNA fragments. cDNA from UC/HeLa 2-5 cells was used as template with the primer pairs A in the sense orientation, 5′-ACN-CAR-GGN-CCN-GAR-CAR-CAR-CA⊕-3′, and C′ in the antisense orientation, 5′-NGT-YTC-NGG-NGT-YTC-NAR-YTG-3′ where N = A, C, G, or T; R = A or G; and Y = C or T. The PCR product was electrophoresed in a 1.5% agarose gel and stained with ethidium bromide. Lane 1 shows the 350-bp amplified product. Lane 2 shows the 350-bp product and other products mentioned in the text. (*B*) Southern blot analysis of pCDM8 cDNA libraries made from UC and UC/HeLa 2-5 poly(A)$^+$ RNA. DNA (10 μg) from UC cell cDNA library (lane 1) and UC/HeLa 2-5 cell cDNA library (lane 2) was digested with *Xba* I, electrophoresed, blotted, and probed with the [^{32}P]dCTP-labeled 350-bp PCR product shown in *A*, lane 1.

and C were selected as primers for PCR from the peptide sequences of TNFR. Since the amino- versus carboxyl-terminal orientation of peptides G522 and G523 to one another was not known, oligonucleotide A was used in the sense and antisense orientation with primers B′ or C′ in the opposite orientation. A PCR-amplified product of ≈350 base pairs (bp) in length was generated only when primer A was used in the sense orientation with primer B′ or C′ in the antisense orientation (Fig. 3*A*, lane 1). This productive combination of primers established that peptide G523 lies amino-terminal to peptide G522 within the TNFR protein. Upon occasion, by using the same A and C′ combination of primers, additional products of the PCR were also observed (Fig. 3*A*, lane 2). These spurious bands are possibly due to the high degeneracy (greater than 1:1000) of the oligonucleotide primer sequences. By including [^{32}P]dCTP during the PCR, a high-specific-activity radiolabeled DNA probe ($2–5 \times 10^9$ cpm/μg) was generated with primers A and C′, which was then used to analyze and screen the pCDM8 UC/HeLa 2-5 cDNA library.

Preferential Hybridization of Radiolabeled PCR Probe to pCDM8 cDNA Inserts from Receptor-Overexpressing Cells. cDNA inserts in the pCDM8 cDNA libraries of UC cells and UC/HeLa 2-5 cells, fractionated by gel electrophoresis, were hybridized to the radiolabeled PCR probe. There was significantly increased hybridization to the cDNA from receptor-amplified cells than from the parental cells, presumably reflecting increased abundance of TNFR mRNA in the UC/HeLa 2-5 cells (Fig. 3*B*). These results also indicate that cDNA clones for the receptor are well represented in this library.

TNFR cDNA Cloning. Of 5×10^5 recombinants screened from the UC/HeLa 2-5 library, a total of 30 clones were isolated by hybridization to the radiolabeled PCR probe. These clones were tested for binding to individual oligonucleotides, and 16 clones that hybridized to all four oligonucleotides (i.e., A, B, C, and the 51-mer D) were selected. Restriction analyses plus cross-hybridization of their cDNA inserts revealed overlapping sequences ranging in insert size from 1.0 to 2.9 kb.

TNFR clone 27 contained a 2.8-kb cDNA, and TNFR clone 16 contained a 2.5-kb insert; together these clones span a length of 3.3 kb. Clone 27 contains *Alu* repeats and the 3′ polyadenylylated end of the mRNA, and clone 16 extends ≈500 bases further than clone 27 towards the 5′ end of the mRNA. Clone 16 was sequenced and contained a single open reading frame encoding 344 amino acids and an in-frame termination codon, TAA. The 3′ untranslated sequence comprises ≈2.3 kb of the combined 3.3-kb length of clones 16 and 27. The deduced amino acid sequence of the open reading frame of clone 16 is presented here (Fig. 4) and contains the sequence for all four receptor peptides. Therefore, this cDNA clone obtained from the purified TNF-receptor appears to encode a partial sequence of the TNFR gene.

Size of the TNFR mRNA. Poly(A)$^+$ RNAs from parental UC cells and UC/HeLa 2-5 cells were analyzed with random hexamer-primed ^{32}P-labeled clone 16 cDNA. A single mRNA species 3.8 kb long was readily identified in UC/HeLa 2-5 RNA preparations, and a longer exposure of the blot to x-ray film revealed a mRNA of the same size in the parental unamplified cells (Fig. 5 *A* and *B*).

Analysis of Genomic DNA for the TNFR Gene. Southern blots of genomic DNA digested with several restriction enzymes showed that UC cells contained single *Kpn* I and *Sph* I restriction fragments hybridizing to the cDNA probe, whereas the *Xba* I digest revealed two bands, possibly caused by the presence of this restriction site within the parental gene. These results are compatible with the presence of a single TNFR gene in these cells. Restriction digests of UC/HeLa 2-5 DNA generate the same bands plus an additional more intense band that presumably reflects amplification of the introduced gene from the EBOpcD-HeLa cDNA library (25). Support for this interpretation comes from parallel experiments with *Eco*RI as the restriction enzyme in which radiolabeled vector sequences hybridized to the same

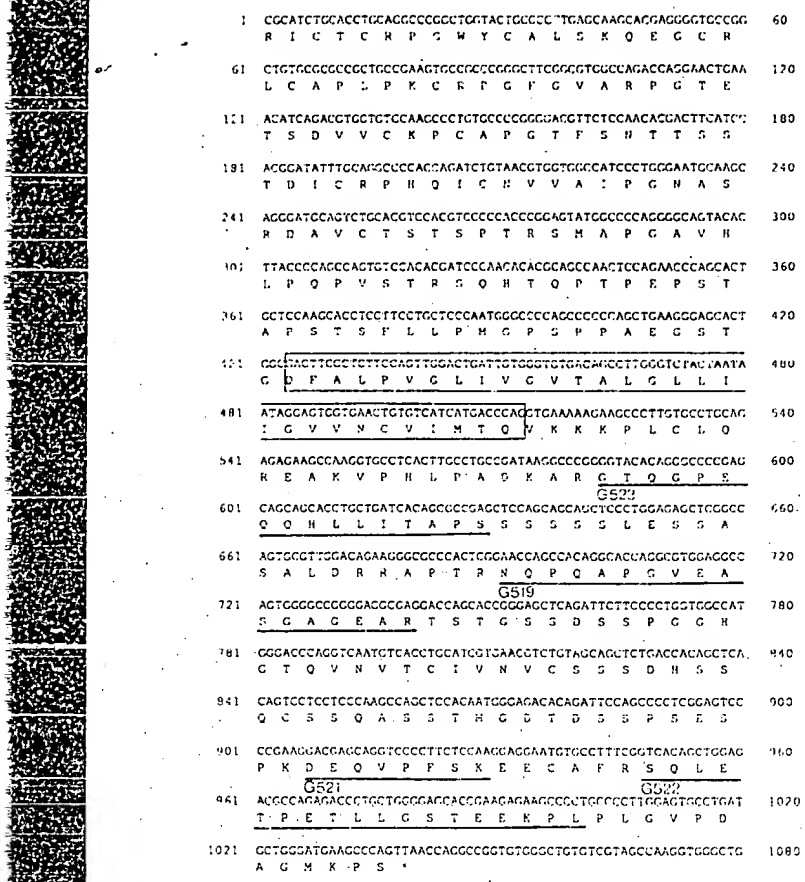

FIG. 4. Coding sequence from the 2.5-kb *Xba* I insert of TNFR clone 16 cDNA. The predicted amino acids encoded by the DNA sequence are shown in single-letter code, and the positions of the peptide sequences derived from the protein are underlined and are consistent with PCR amplification and ordering. The sequence shown indicates the stop codon, TAA, with a star.

intense band as the cDNA probe (data not shown). We surmise that this amplified sequence confers high-level TNFR expression to these cells.

DISCUSSION

Recently, we described the purification of TNFR protein (25). Four peptide sequences—8, 16, 17, and 18 amino acids long—were derived from tryptic digests of this protein (Fig. 1). This report describes the cloning of a TNFR cDNA based on these partial protein sequences. The cDNAs were isolated with PCR probes prepared with oligonucleotide primers corresponding to the amino acid sequence of two tryptic peptides. Of the 16 positive clones isolated, further analysis by restriction mapping and DNA sequencing of two cDNAs, clones 16 and 27 provided a total of 3.3 kb of DNA sequence. This sequence contains the 3′ end of the mRNA as evidenced by the presence of a poly(A) tail and also contains *Alu* repeated sequences (data not shown). A single open reading frame (encompassing 344 amino acids) followed by an in-frame termination codon is presented in this report. The deduced amino acid sequence from the open reading frame (Fig. 4) contains the sequence of all four receptor peptides derived from the purified protein. Therefore, this sequence was verified as a partial cDNA sequence encoding the TNFR. A hydropathy plot (43) of the coding sequence reveals a major hydrophobic domain of 30 amino acids (residues 141–170), presumably the transmembrane sequence, that separates a

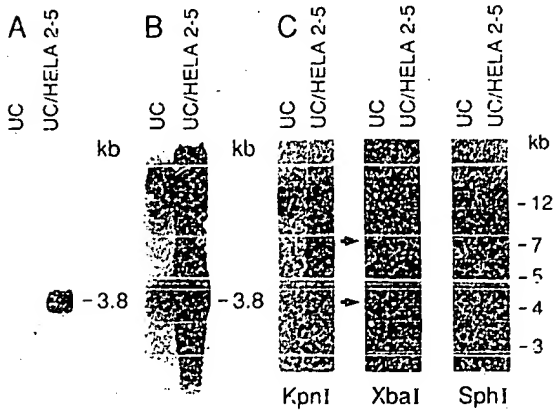

FIG. 5. Analyses of mRNA and genomic DNA encoding the TNFR. Northern blot analysis of poly(A)$^+$ RNA (10 μg per lane) from parental UC cells and UC/HeLa 2-5 cells was conducted as described with [^{32}P]dCTP-labeled TNFR clone 16 cDNA as a probe. Autoradiograph exposure times were 1 hr (*A*) and 16 hr (*B*). (*C*) Genomic Southern blot analysis. DNA (10 μg) was digested separately with restriction endonucleases (*Kpn* I, *Xba* I, and *Sph* I), electrophoresed, blotted, and probed with a [^{32}P]dCTP-labeled 270-bp fragment from the 5′ end clone 16 cDNA. Arrows indicate the positions of the hybridizing bands.

174-amino acid intracellular carboxyl-terminal signaling domain from an extracellular amino-terminal ligand-binding domain. Northern blot analysis showed an ≈3.8-kb mRNA present in UC/HeLa 2-5 and the parental UC cells. This observation implies that the cDNA sequence we have obtained is ≈500 bp short (at the 5′ end) of a full-length clone. Not accounting for the lengths of the 5′ untranslated and leader sequence, the receptor contains a maximum of 170 amino acids in addition to the 344 residues represented in clone 16. The mature TNFR would then be no more than 514 amino acids with a molecular mass of 57 kDa. This is a smaller size than the 68-kDa estimate obtained by PAGE, but the apparent discrepancy is most likely due to glycosylation of the protein.

The successful use of primers A and C in generating a PCR fragment allowed certain predictions about the TNFR protein sequence: (*i*) that peptide G523 is located amino-terminally with respect to peptide G522, and (*ii*) that ≈350 bp lie between their respective coding sequence. These predictions held true, and 387 nucleotides were found to cover the distance from A to C (Fig. 4). Generation of radiolabeled probes with PCR (39), as used here, has provided distinct advantages over screening libraries with oligonucleotides. The increase in the length of the probe by the added amplified DNA fragment and the generation of high-specific-activity DNA (2–5 $\times$ 10^9 cpm/μg) by incorporation of radioisotope during the amplification reaction have allowed stringent hybridization and wash conditions to be performed that eliminate high backgrounds and false positives as seen with the unstable hybrids made by short oligonucleotides.

Amino-terminal sequence analysis revealed heterogeneities, since several amino acids were detectable in each cycle. The result is compatible with the sequence Val-Ala-Phe-Thr-Pro found in the recently published (24) urinary TNF-binding protein II, which was also reported to be variable at the amino terminus. These comparisons implied a possible relatedness of the TNFR with TNF-binding protein II and the likelihood that the soluble protein was derived from the receptor. Upon analysis of the growth medium from TNFR-overexpressing cells, TNF-binding activity was readily observed, and after ligand blot analysis, a major and minor form of ≈40 kDa and 30 kDa, respectively, were seen. The size of the predominant form is larger than the 30-kDa estimate reported for the

urinary protein and suggests that further proteolysis of the 40-kDa form may occur during its passage through the serum or urine *in vivo* to generate the smaller molecule. If the extracellular domain of the TNFR comprised a maximum of 310 amino acids (≈34 kDa), then the soluble 40-kDa form either could be a product of a different mRNA or could be glycosylated, with the linked carbohydrates accounting for the difference in size. A comparative sequence analysis of the two proteins should provide further information regarding their structural relationships.

Soluble forms of cell associated receptors are generated both by differential splicing of the receptor mRNA (44–46) and by the action of proteases on extracellular domains of the receptor (47, 48). The mechanism(s) for the production of the shed form of TNFR is presently not known; however, the degree of overexpression of mRNA and the abundant production of the TNFR by these cells should facilitate analysis of this phenomenon. The physiological significance of soluble receptors can at best be only surmised. They may provide a protective mechanism against systemic activity of these potent cytokines or a mechanism for their elimination or both. Possibly the low abundance of cytokine receptors may be explained in part by their shedding from cell surfaces (49).

UC/HeLa 2-5 cells were developed by transfection of HeLa cDNA libraries into UC cells (25). We have assumed that increased receptor production is associated with an introduced copy of the TNFR coding sequence. Results from genomic Southern blots presented here show that while the parental gene in UC cells is unaltered, a second very intense band of TNFR is seen only in UC/HeLa 2-5 cells. This may represent the introduced cDNA that upon amplification confers high-level TNFR expression.

After this paper was submitted for publication, two reports on the cloning of a 50- to 55-kDa TNFR were published (50, 51), the sequence of which is different from the 68-kDa TNFR reported here. In addition to the difference in size of the mature proteins and the mRNA transcripts encoding them, the calculated identity based on protein sequences and the BestFit alignment of Smith and Waterman (52) is 21.7%. Therefore, these proteins appear to represent two unique TNFRs that give rise to the two reported (24) urinary TNF-binding proteins.

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Amplified Expression of Tumor Necrosis Factor Receptor in Cells Transfected with Epstein-Barr Virus Shuttle Vector cDNA Libraries*

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As an approach to isolate the cell-surface receptor for tumor necrosis factor (TNF), we have developed transfectants of human B-lymphoblastoid cells (UC cells) that overexpress the TNF receptor. These transfectants were isolated from UC cells transfected with cDNA libraries of HeLa or NG108 cells constructed in the mammalian expression vector EBO-pcD. This vector contains the Epstein-Barr virus origin of replication (*ori*-P) plus the EBNA-1 gene conferring replication function to *ori*-P and, therefore, the ability to replicate autonomously within the transfected cell (Margolskee, R. F., Kavathas, P., and Berg, P. (1988) *Mol. Cell. Biol.* 8, 2837–2947). Cells overexpressing the TNF receptor were identified and separated by the binding of fluoresceinated TNF and flow cytometric selection. Scatchard analysis of ^{125}I-TNF binding data revealed a single class of high affinity receptors with a dissociation constant (K_d) of 0.2 to 2 nM and a receptor density of about 150,000 per cell, an increase of approximately 150-fold over UC cells. Cross-linking of receptor-ligand with bis-sulfosuccinimidyl suberate followed by polyacrylamide gel electrophoresis gave estimates of 87 and 104 kDa for the size of the complex. Based on its ability to bind TNF, a 68-kDa receptor protein was identified in cell extracts enriched for the receptor by using immobilized wheat germ agglutinin and TNF affinity chromatography. The difference in the estimated size of the receptor and the receptor-ligand complexes demonstrates that TNF binds to the receptor as a monomer or a dimer.

Analysis of cDNA sequences conferring receptor amplification in transfectants revealed that plasmid DNA was present at 30 or more copies per cell, most likely integrated into the genomic DNA or organized into high molecular weight catenanes, and autonomously replicating units could not be recovered. Therefore, while this vector was useful in generating stable receptor-amplified cells, it was not maintained as a recoverable episome.

Tumor necrosis factor (TNF)[1] is a 17-kDa polypeptide secreted by macrophages and a member of the immunoregulatory class of molecules termed cytokines that includes factors such as interleukins and interferons. Like other cytokines, TNF exerts a diversity of biological responses, including inflammation (1–3), endotoxin shock (4), cytotoxicity to certain tumors and tumor cell lines (5, 6), and the catabolic state termed cachexia associated with chronic disease (7, 8).

TNF interacts with a wide variety of cell types by binding to high affinity receptors present on the cell surface. The availability of radioiodinated derivatives of TNF has made it possible to measure the number of surface receptors on many different cell types. Scatchard analyses of the ligand-receptor binding data indicate that the number of receptors is small, usually in the range of 500 to 10,000 per cell (7, 9–12). Higher numbers (around 50,000 per cell) have been reported for HeLa-S_3 (11) and a subcloned human histiocytic cell line U937 (13), although these have not been confirmed by other[2]. There appears to be a single class of specific high affinity receptors with affinity constants for binding in the 0.1 to 10 nM range (11, 12, 14, 15). Ligand-receptor complexes are thought to be internalized, processed, and degraded in the lysosomes (11, 16–18). Interleukin-1 (19) and tumor promoters such as phorbol esters (19–22) down-regulate receptor numbers while interferon-γ enhances receptor expression (15, 17, 23). Although little is known about post-receptor events leading to the production of intracellular messengers, G-proteins have been implicated in the signal transduction pathway by the demonstration that pertussis toxin inhibits TNF-induced cytotoxicity (24). TNF also stimulates arachidonic acid release and subsequent production of prostaglandins (25).

One difficulty in the isolation of the receptor protein has been the low abundance of TNF receptors on cells. The protein has been partially purified from human histiocytic cells (U937) and estimated to have a size of 65 ± 32 kDa (13). However, extremely low recovery plus the presence of other proteins make this size estimate tentative. Covalent cross-linking of radioiodinated TNF to receptor with bis-sulfosuccinimidyl suberate generates products ranging from 74 to 105 kDa (11, 13, 16), but with 1,5-difluoro-2,4-dinitrobenzene (a different cross-linking reagent) two additional complexes of 54 and 138 kDa were reported (26). The size of the receptor itself from this ligand-receptor complex is difficult to derive because of the uncertainty of the form of TNF associated with the receptor. TNF self-associates predominantly as a trimer (27–91%) but its interaction with the receptor as a monomer,

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[1] The abbreviations used are: TNF, tumor necrosis factor-α; TNF-R, TNF receptor; FITC-TNF, fluorescein isothiocyanate-conjugated TNF; EBV, Epstein-Barr virus; UC cells, EBV-transformed human B-lymphoblastoid UC729-6 cells; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; PBS, phosphate-buffered saline; WGA, wheat germ agglutinin; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; kb, kilobase pairs; FACS, fluorescence activating cell sorting.

[2] HeLa-S3 cells were obtained from the American Type Culture Collection, Rockville, MD, and when used to measure TNF receptor number had approximately 3000 receptors per cell but no more than the HeLa cells used in this investigation.

dimer, or trimer has not been established. A full understanding of TNF-receptor interaction requires the isolation and identification of the receptor, characterization of its ligand binding properties, and elucidation of the intracellular mechanisms by which it asserts its cytotoxic action and other biological responses.

As an approach to the isolation of the receptor, we report the development and use of human B-cell lines that overexpress TNF receptor (the receptor is defined here as a cell-surface protein that binds TNF with high affinity). These cells were isolated from a pool of B-cells transfected with cDNA libraries, derived from HeLa or NG108 cells, constructed in the vector EBO-pcD. This vector permits expression of insert cDNA in mammalian cells, transforms human cells with high efficiency, and has the potential to exist as an autonomous plasmid at 2–10 copies within the transfected cell. Recovery of introduced cDNA clones from mammalian cells should therefore be facilitated (29). However, in this case the EBO-pcD vector appears either to integrate into cellular genomic DNA or to exist as a high molecular weight concatamer not recoverable as plasmids. We have nevertheless utilized these cell lines to identify the TNF receptor in detergent extracts of cells after enrichment for the receptor using affinity chromatography procedures. Based on the size of the receptor and the receptor-ligand complexes we conclude that TNF associates with the receptor primarily as a monomer and to a lesser extent as a dimer.

EXPERIMENTAL PROCEDURES

Materials—Carrier-free $Na^{125}I$ was purchased from Du Pont-New England Nuclear; IODO-GEN, bis-sulfosuccinimidyl suberate, NHS-LC-biotin, and Triton X-100 were from Pierce Chemical Co.; penicillin, streptomycin, RPMI 1640, Dulbecco's modified Eagle's medium, and fetal calf serum were from GIBCO; complete RPMI 1640 and RPMI 1640 without phenol red or biotin was from Irvine Scientific, Santa Ana, CA. All restriction enzymes and hygromycin B were purchased from Boehringer Mannheim; fluorescein isothiocyanate was purchased from Molecular Probes, Eugene, OR; anti-Leu-2a (CD8) was from Becton-Dickinson, Mountain View, CA; BA85 nitrocellulose membrane was from Schleicher & Schuell; *N*-acetylglucosamine, phenylmethylsulfonyl fluoride (PMSF), pepstatin, leupeptin, and aprotinin were from Sigma; wheat germ lectin and CNBr-activated Sepharose 4B were from Pharmacia LKB Biotechnology Inc.; and protein silver stain was from Bio-Rad.

Cell Culture—NG108 cells (NG108-15 rat neuroblastoma × mouse glioma hybrid cells) were obtained from Dr. James Eberwine, Department of Psychiatry, Stanford University; 293S human embryonic transformed kidney cells were from Dr. Michele Calos, Department of Genetics, Stanford University; EBV-transformed human lymphoblastoid UC 729-6 cells (UC cells), HeLa cells (human epithelioid cervical carcinoma cells), and COS-7 (SV40-transformed CV1 African green monkey kidney cells) were obtained from Dr. Paul Berg's laboratory, Stanford University. HeLa cells, NG108 cells, COS-7 cells, and 293S cells were maintained in Dulbecco's modified Eagle's medium while UC cells were cultured in RPMI 1640 medium. Media were supplemented with penicillin and streptomycin plus 10% fetal calf serum. Hygromycin was added to 200 μg/ml concentration. Cells were maintained in 5% CO_2, 95% air in 37 °C.

EBO-pCD HeLa and NG108 Expression Libraries—Expression libraries and their size-selected fractions were prepared in the pcD expression plasmid developed by Okayama and Berg (30) with modifications for insertion of the Epstein-Barr virus sequences as described by Margolskee *et al.* (29).

Radioiodination of TNF—Purified recombinant TNF was provided by Cetus Corp., Emeryville, CA. A 4-μg aliquot in 100 μl of 0.1 M NaH_2PO_4 buffer, pH 7.4, was mixed with 1 mCi of carrier-free $Na^{125}I$ (Du Pont-New England Nuclear), in 3 μg of IODO-GEN-coated glass tube for 5 min at room temperature. The reaction was brought up to 1 ml with the buffer containing 0.1% gelatin (PBS buffer), and the unreacted iodine was removed by gel filtration on a Sephadex G-25 PD-10 (Pharmacia LKB Biotechnology Inc.) column equilibrated with PBS buffer. The column was washed with 1.9 ml of buffer and the flow-through discarded. Radioiodinated TNF was eluted with the next 1.5 ml of buffer. The specific activity of the product was 20 μCi/μg TNF. More than 95% of the ^{125}I was incorporated into TNF as determined by trichloroacetic acid precipitation of total radioactivity and by SDS-PAGE in which a single band of TNF at 17 kDa was detected as radiolabeled.

^{125}I-TNF-Receptor Binding Assay—Binding assays on intact cells in suspension were performed in 200 μl of RPMI 1640 medium supplemented with 10% fetal calf serum. One × 10^6 cells were incubated on ice for 2 h with various concentrations of ^{125}I-TNF with or without 100-fold excess of cold TNF. Cells were washed three times with ice-cold culture medium, and radioactivity in the cell pellet was determined in a gamma counter. Specific binding is defined as the amount of ^{125}I-TNF that can be displaced by 100-fold excess cold TNF. Soluble receptor was identified by a filter binding assay to immobilize proteins on a nitrocellulose membrane held in a Minifold II apparatus (Schleicher & Schuell). The membrane was air-dried after sample application and blocked in a 5% non-fat dry milk solution made in phosphate-buffered saline (PBS) for 2 h at room temperature. Immobilized receptor was incubated with ^{125}I-TNF at a 10 ng/ml concentration in 10% fetal calf serum in PBS for 1 h. The filter was washed three times for 15 min each at room temperature with PBS followed by autoradiography. This assay is described here as a method of tracing receptor activity. Details of it as a quantitative receptor assay will be published elsewhere.

Fluorescein or Biotin Conjugation of TNF—TNF (10 mg/ml) dissolved in sodium bicarbonate buffer, pH 9.5, was conjugated to fluorescein isothiocyanate (FITC) dissolved in dimethyl sulfoxide. A 290-μg aliquot of TNF was added to 66 μg of FITC in a total volume of 35 μl, and the vial containing the reagents was wrapped in aluminum foil and rocked gently at room temperature for 2–3 h. A PD-10 column was equilibrated with PBS buffer and the FITC-TNF solution brought to 100 μl with PBS buffer was loaded onto the column. The column was washed with 1.9 ml of buffer and the flow-through discarded. FITC-TNF was collected with the next 1.5 ml of buffer eluate. NHS-LC-biotin (10 mg/ml) in dimethyl sulfoxide was reacted with TNF (1 mg/ml) in 0.1 M bicarbonate buffer, pH 8.5, at a 2:1 molar ratio at room temperature for 2 h. Dimethyl sulfoxide in the reaction mixture was less than 10% of the total reaction volume. Biotinylated TNF was recovered by gel filtration as described above for FITC-TNF.

Transfection of Human Lymphoblastoid Cells—UC cells (1×10^8) were electroporated separately with 50 μg of cDNA from each library and 20 μg of EBO-pcD Leu 2. Conditions for cell preparation, electroporation, and selection of hygromycin-resistant populations were as described previously (29).

Flow Cytometry and Sorting—Transfected UC cells (2×10^6) were prepared for flow cytometry by staining with FITC-TNF at a concentration of 850 ng/ml in 800 μl of RPMI-deficient medium at 4 °C for 2 h. Propidium iodide (Calbiochem) was added at a final concentration of 5 μg/ml prior to analysis to label dead cells. Cells were analyzed and sorted on a FACStar Plus sorter (FACS II, Becton Dickinson, San Jose, CA) by illuminating them with 500 milliwatts of 488 nm laser light and measuring forward and wide angle light scatter, 530 nm FITC fluorescence, and propidium iodide fluorescence greater than 630 nm. Sort windows were generated to eliminate debris and to sort the brightest 1% of FITC fluorescence distribution which was propidium iodide-negative. Early selection was accomplished using Becton-Dickinson's "enrich" mode, which accepts coincident particles in positive sort envelopes. Approximately 50,000 cells were grown in culture to 1–2 × 10^6 cells in the presence of 200 μg/ml hygromycin, and this cycle of selection and expansion was repeated as described.

Cross-linking of ^{125}I-TNF to Receptors—Cells (1–2 × 10^6) were incubated for 2 h at 4 °C in RPMI 1640 plus 10% fetal calf serum (culture medium) containing 300 ng/ml of ^{125}I-TNF with or without 100-fold excess of unlabeled TNF. After incubation, the cells were washed three times by centrifugation through ice-cold culture medium to remove unbound TNF and resuspended in 1 ml of PBS. To this suspension 20 μl of 50 mM bis sulfosuccinimidyl suberate in PBS was added, and the cells were left at room temperature for 30 min. The cells were washed three times with PBS and lysed in 0.5% Nonidet P-40. Undissolved debris was removed by centrifugation at 10,000 × *g* for 5 min, and the protein in the supernatant was precipitated with 10% trichloroacetic acid. The pellet was resuspended in 50 μl of Laemmli sample buffer and the proteins were analyzed on 10% polyacrylamide gels according to the method of Laemmli (31). After electrophoresis, the gels were dried and subjected to autoradiography.

Solubilization and Enrichment of the Receptors—Approximately 1 × 10^{10} UC/HeLa 2-5 cells or UC cells grown in roller bottles were

solubilized in 200 ml of cold extraction buffer containing 1% Triton X-100, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM PMSF, and 10 μg/ml each of pepstatin, leupeptin, and aprotinin. The insoluble material was removed by centrifugation at 100,000 × *g* for 1 h, and the extract was enriched for the receptor by using two sequential affinity chromatography steps. The extract was first added to a 60-ml column of wheat germ agglutinin (WGA) coupled to Sepharose 4B (15 mg of WGA coupled to 1 g of Sepharose 4B purchased from Pharmacia LKB Biotechnology Inc and prepared according to directions provided by the vendor). The column was washed with 10 column volumes of column buffer containing 0.2% Triton, 50 mM Tris-HCl, pH 7.4, 0.5 M NaCl plus protease inhibitors described above. The column was eluted with 3 column volumes of the column buffer containing 0.5 M *N*-acetylglucosamine. The eluates were dialyzed against cold PBS containing 0.2% Triton and added to a 5-ml column of TNF coupled to Sepharose 4B (3.5 mg of TNF per g of Sepharose 4B). The column was washed sequentially with 10 column volumes of column buffer and then 0.2 M glycine HCl, pH 4.0, plus 0.2% Triton and protease inhibitors. The receptor was eluted with glycine HCl, pH 2.5, containing 0.2% Triton and protease inhibitors. Eluates were concentrated with a Centricon filter (Amicon, Danvers, MA) and analyzed for receptor activity and protein content.

Ligand Blot Analysis of Protein with TNF Receptor Activity—Specific amounts of protein were resolved by electrophoresis in a 10% polyacrylamide gel run at 4 °C according to the procedure of Laemmli (31) except that no sulfhydryl reducing agents were added and samples were not heated prior to the run. After electrophoresis the samples were electroblotted at 4 °C onto nitrocellulose membrane in buffer systems described by Burnette (32). The nitrocellulose membrane was incubated in 8 M urea, 0.2% Triton X-100, 50 mM Hepes, pH 7.4, for 1 h, blocked with 5% non-fat dry milk/PBS solution, and incubated with ^{125}I-TNF, washed and analyzed as described for the soluble receptor assay.

Isolation of Episomal DNA from Transfected Cells—Episomal DNA from UC cell transfectants was isolated from 2 × 10^7 cells according to the method of Hirt (33). Cells were lysed in 0.6% SDS, 1 M NaCl, 10 mM Tris, pH 7.5, and 10 mM EDTA and kept at 4 °C overnight. High molecular weight DNA was removed by centrifugation at 16,000 × *g* for 30 min at 4 °C. The supernatant was treated with proteinase K for 1 h at 65 °C, extracted with phenol/$CHCl_3$ (1:1), and the DNA precipitated by addition of 2.5 M ammonium acetate plus 2 volumes of absolute ethanol. After overnight storage at −20 °C, the DNA was pelleted by centrifugation at 16,000 × *g* for 30 min at 4 °C, washed with 80% ethanol, dried, and resuspended in 20 μl of 10 mM Tris, and 1 mM EDTA, pH 7.5. Aliquots of this DNA were used to transform competent *Escherichia coli* cells (DH5) purchased from Bethesda Research Laboratories, according to instructions provided by the vendor. Bacterial cells (100 μl) and DNA were incubated for 30 min on ice, heat-shocked for 45 s at 42 °C, and cooled on ice for 2 min. SOC medium (0.9 ml) (SOC medium: LB plus 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) specified by the vendor was added to this mixture, and cells were shaken at 225 rpm for 1 h at 37 °C. Cells were plated on LB medium plates containing 50 μg/ml ampicillin, and transformants were recovered after an overnight incubation at 37 °C. Individual bacterial colonies were grown in 2-ml cultures in LB plus ampicillin. Plasmid DNA was prepared according to the method of Holmes and Quigley (34) and the extracted DNA analyzed by restriction endonuclease digestion and agarose gel electrophoresis.

Southern Blot Analyses—Episomal DNA and genomic DNA prepared from transfected UC cells were analyzed according to published procedures (35). After digestion with restriction endonucleases, DNA was electrophoresed in 0.8% agarose gels and transferred to nitrocellulose membranes (Schleicher & Schuell). These membranes were hybridized with ^{32}P-labeled, random hexamer-primed probes (36), and the hybridized bands were observed by autoradiography. Recovery of episomes from transfected cells proved to be difficult. Possible reactions that could be offered are the formation of concatamers from introduced vector-cDNA sequences, rearrangements between introduced and host DNA, and/or integration into genomic DNA. A loss of one SV40 promoter plus introduction of Alu repeated sequences (36) were some of the rearrangements noted in genomic DNA preparations analyzed from the transfectants (data not shown).

RESULTS

By using ^{125}I-TNF we found that a variety of cell lines (*e.g.* HeLa and NG108) contain approximately 3000 TNF receptors per cell whereas the UC, B-lymphoblastoid line, contains about 1000 receptors per cell (Fig. 1). In each case a single class of high affinity receptors was identified with a K_d for TNF between 0.1 and 1.0 nM. Attempts to isolate a functional cDNA encoding the TNF receptor were initiated based on the notion that UC cells expressing additional receptor from an exogenously introduced cDNA could be identified by one of several techniques. Consequently, we prepared cDNA expression libraries in the Epstein-Barr virus shuttle vector, EBO-pCD (Fig. 2), using RNA from HeLa and NG108 cells (29). The HeLa libraries were made from five different sizes of cDNA in which the cDNA inserts were size 1, <0.4 kb; size 2, 0.4–0.8 kb; size 3, 0.8–1.6 kb; size 4, 1.6–2.9 kb; and size 5 with greater than 2.9-kb cDNA inserts. Fractions 2 through 5 alone or a pool of these four fractions referred to as HeLa 2-5 or the NG108 total cDNA library were introduced separately into UC cells by electroporation of 1 × 10^8 cells with 50 μg of library DNA. EBO-pcD Leu 2, harboring a cDNA encoding a human T-cell surface marker, was transfected separately into a population of UC cells to monitor the efficiency of electroporation, hygromycin selection, and flow cytometric analysis (29). Transformed cells were recovered by their resistance to hygromycin and required approximately 2 weeks of growth in drug-containing medium for selection. The expression of TNF receptor on these cells was measured by analyzing the binding of fluoresceinated TNF in a flow cytometer.

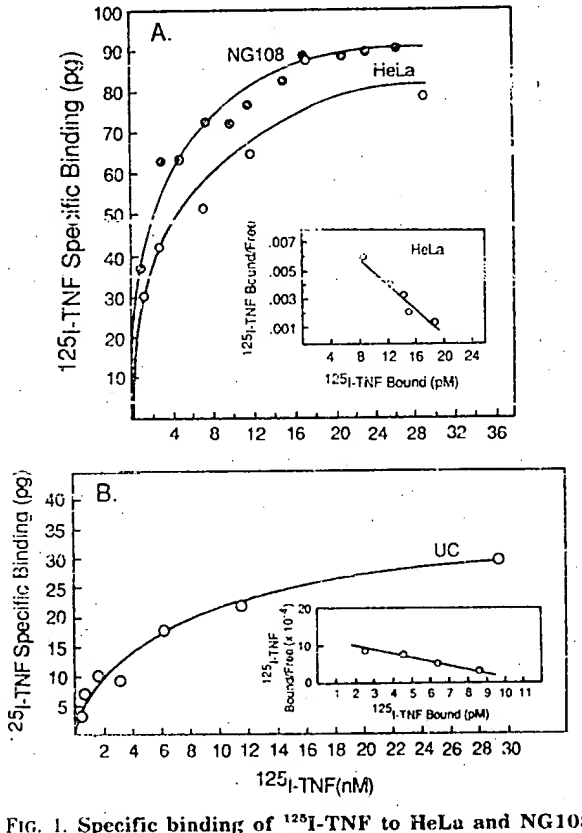

FIG. 1. **Specific binding of ^{125}I-TNF to HeLa and NG108 (*A*) and UC cells (*B*).** One × 10^6 cells were incubated at 4 °C for 2 h with increasing amounts of ^{125}I-TNF in the absence or presence of 100-fold excess unlabeled TNF. Cells were then washed three times and the amount of bound ligand determined by counting cell-associated radioactivity. The estimated number of receptors and the dissociation constants (K_d) calculated from Scatchard analyses (*inset*) are: UC cells 1300 receptors/cell, $K_d = 0.9 \times 10^{-9}$ M; HeLa cells, 2900 receptors/cell, $K_d = 2 \times 10^{-9}$ M; and NG108 cells, 3400 receptors/cell.

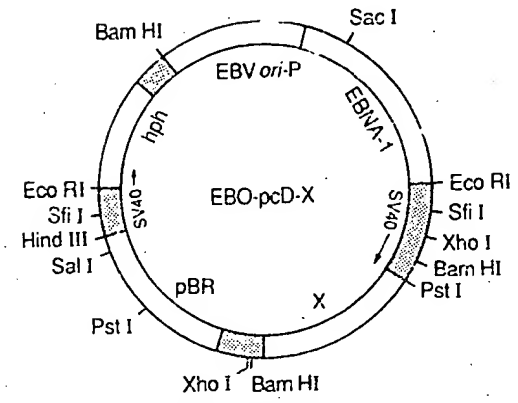

FIG. 2. **Structure of the EBO-pcD plasmid.** The construction and structure of the EBO-pcD plasmid were published earlier (29) and are described here to simplify presentation of data. In the diagram the lower half of the circle as defined by the two *Sfi*I sites represents a pcD-cDNA plasmid according to Okayama and Berg (30). It contains the SV40 region of DNA replication and the early region promoter transcribing cDNA in the clockwise direction. It is joined to a fragment containing the 19 S and 16 S SV40 late region mRNA intervening sequence. × depicts the position of any cDNA in the library and at its 3′ end × is linked to the SV40 late region polyadenylation signal. The next fragment is derived from pBR322 and carries the origin of replication for propagation in bacteria plus the β-lactamase gene to confer resistance to ampicillin. The *Sfi*I fragment of DNA in the upper half of the circle contains a cassette of genes required to convert the pcD-vector into the EBO-pcD plasmid. It contains the hygromycin-phosphotransferase gene (*hph*) transcribed by the SV40 early region promoter and at its 3′ end SV40 small t-antigen intervening sequence plus polyadenylation signal; the EBV origin for plasmid replication *ori*-P in EBV-transformed cells and the EBNA-1 gene are linked to the hygromycin construct. EBNA-1 is transcribed in the counterclockwise direction presumably by the SV40 late region promoter. All SV40 sequences in the diagram are represented as *stippled areas*. The vector contains two SV40 early promoter sequences oriented in the clockwise direction. The relevant restriction sites defining components of this vector are indicated. Approximate lengths of different restriction fragments as determined by comigrating of DNA size markers in agarose gel electrophoresis are: 2.6 kb for *Xho*I at the 3′ end of the cDNA insert to *Hin*dIII; 2.5 kb from *Hin*dIII to *Bam*HI located at the 3′ end of the hygromycin gene; 3.1 kb for the *Bam*HI to *Sac*I site; and 1.9 kb from *Sac*I to *Eco*RI site adjacent to the SV40 promoter driving cDNA expression.

Selection of UC Transfectants Expressing Elevated Levels of TNF Receptor—In order to detect the rare cells expressing higher receptor number, two derivatives of TNF were prepared for fluorescent staining of cells for FACS analysis and tested for their specificity of staining. These derivatives were biotinylated TNF for use with a second step reagent such as FITC-avidin or phycoerythrin-streptavidin and fluorescein-conjugated TNF alone. Class A reagents yielded nonspecific staining, which was 5-fold higher than unstained UC cells and therefore could not be used to measure specific binding of TNF (data not shown). However, FITC-TNF showed little or undetectable levels of nonspecific staining and was useful in detecting receptors on HeLa cells (Fig. 3). Furthermore, FITC-TNF binding was completely displaced by a 100-fold excess of TNF to the level of unstained cells as measured by fluorescence intensity. The fluorescence profile obtained with UC cells (not shown), stained or unstained, was very similar to that of HeLa cells, showing essentially that these cells had very little autofluorescence or nonspecific staining with FITC-TNF. In tests of bioactivity FITC-TNF was comparable with TNF in its cytotoxicity to L929 cells (data not shown), suggesting that little or no loss of TNF function had occurred due to modification with FITC.

UC cell transfectants resistant to 200 μg/ml hygromycin

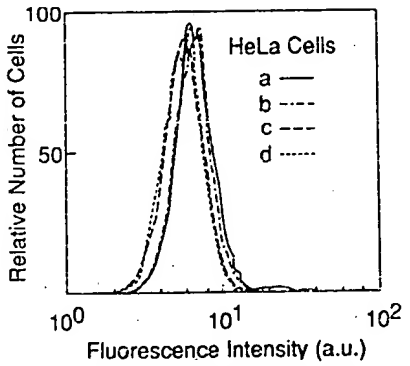

FIG. 3. **Specific binding of FITC-TNF to HeLa cells.** Cells were incubated at 4 °C for 2 h with FITC-TNF in the absence or presence of 100-fold excess TNF. Cells were washed with RPMI-deficient medium, and fluorescence intensity was measured flow cytometrically. *a*, FITC-TNF 0.8 μg/ml; *b*, FITC-TNF 1.5 μg/ml; *c*, FITC-TNF 0.8 μg plus TNF 8 μg/ml; and *d*, unstained HeLa cells. No significant difference in fluorescence intensity was observed between the two different FITC-TNF concentrations tested. Unstained cells and cells incubated with 100-fold excess TNF exhibited overlapping fluorescence curves indicating that all fluorescence due to FITC-TNF could be displaced by TNF. *a.u.* denotes arbitrary units. The very small differences shown are highly reproducible.

were stained with FITC-TNF and analyzed flow cytometrically. Initially, the spectrum of fluorescence intensity in a population of FITC-TNF-stained UC transfectants compared with nontransfected cells was indistinguishable. Nevertheless, about 0.05% of the most intensely staining UC transfectants (about 50,000 cells) were collected ascetically and cultured to grow to a population of 1–2 × 10^6 cells. These cells were restained with FITC-TNF and resorted. By the end of the fifth such selection, a population of cells with distinctly higher FITC-TNF binding was seen in transfectants from the entire HeLa library cDNA, and in the size cut 4 cDNA (representing the 1.6- to 2.9-kb size), and in transfectants from the NG108 cDNA library. HeLa cDNA in the size range of 0.4–0.8, 0.8–1.6, or larger than 2.9 kb showed little enhancement of TNF receptor expression as did the EBO-pcD-Leu 2 plasmid DNA after equivalent repeated rounds of cell sorting (Fig. 4).

The transfectants were characterized further by several independent methods to verify the increase in receptor expression. Measurements made by ^{125}I-TNF binding confirmed flow cytometric observations (Fig. 5). Scatchard plots of the steady-state binding of ^{125}I-TNF to the three positive cell lines revealed the presence of approximately 150,000 TNF receptors with K_d in the 0.2 nM (Fig. 6) as compared with the 1,000 (or fewer) receptors per untransfected UC cell. ^{125}I-TNF binding to the negative transfectants UC/HeLa 5, UC/HeLa 3, or UC/Leu 2 was not significantly different from that of untransfected UC cells.

Size of Cross-Linked Receptor-Ligand Complexes—An estimate of the molecular size of the TNF-receptor complex in amplified receptor cell lines and the parental UC cells was made by cross-linking cell-bound ^{125}I-TNF to its receptors with the reagent bis-sulfosuccinimidyl suberate. The cross-linked receptor-ligand complex was solubilized with Nonidet P-40 and analyzed by SDS-PAGE. Preparations of receptor amplified cells showed the complex as two bands on autoradiographs of the gel: a dense band at 87 kDa and a fainter band at 104 kDa when cells were incubated with ^{125}I-TNF alone, but not when excess unlabeled TNF was included during the binding reaction (Fig. 7). The difference in size between the two bands is approximately 17 kDa and is pos-

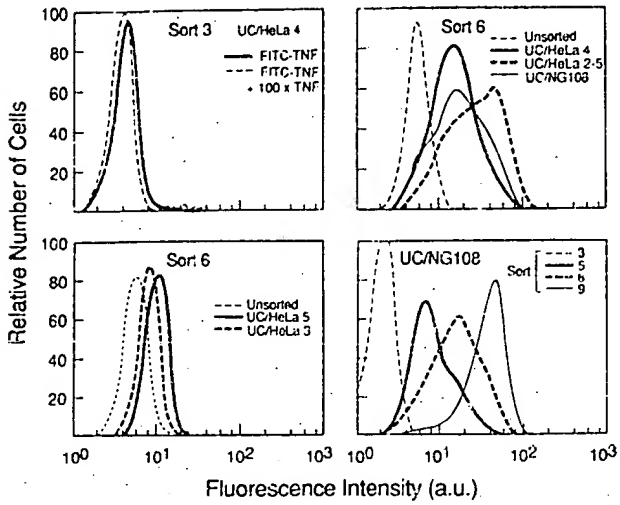

FIG. 4. **Flow cytofluorometric analysis of UC cell transfectants.** Unselected B-lymphoblastoid UC cells (UC/unsorted) or UC transfectants obtained by electroporation with DNA from with either EBO-pcD HeLa size-selected libraries UC/HeLa 2 with 0.4–0.8-kb cDNA; UC/HeLa 3 with 0.8–1.6-kb cDNA; UC/HeLa 4 with 1.6–2.9-kb cDNA; UC/HeLa 5 containing greater than 2.9-kb cDNA; or a pool of all four sizes referred to as UC/2.5; or the UC/NG108 cDNA library were selected for hygromycin resistance and stained with 0.8 μg/ml FITC-TNF and analyzed. *Upper left panel*, UC/HeLa 4 transfectants stained with 0.8 μg/ml FITC-TNF in the absence or presence of 8 μg of TNF to demonstrate detectable TNF receptor after three rounds of flow selection. *Upper right panel*, UC/uns (control) plus UC-transfectants after six repeated rounds of sorting to show amplifications of TNF-R determined by increase in fluorescence intensity due to increased FITC-TNF binding. These are UC/HeLa 4, UC/HeLa 2-5, and UC/NG108. *Lower left panel*, UC/uns (control) plus UC transfectants UC/HeLa 3 and UC/HeLa 5 showing very small increases in TNF-R after six selection cycles. *Lower right panel*, UC transfectants derived from NG108 cDNA library after three, five, six or nine repeated rounds of sorting to show the progression in TNF-R amplification. *a.u.* denotes arbitrary units.

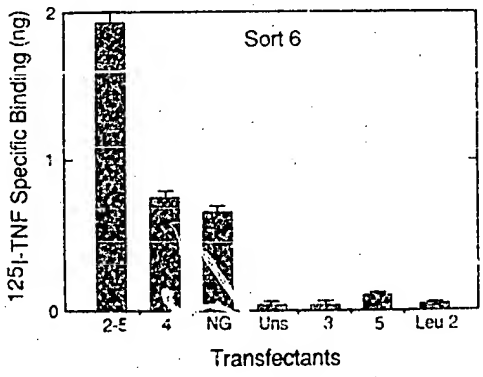

FIG. 5. **Equilibrium specific binding of ^{125}I-TNF to UC cell transfectants.** Binding to UC/HeLa 2-5 (*2-5*), UC/HeLa 4 (*4*), UC/NG108 (*NG*), UC/uns control cells (*Uns*), and UC/HeLa 3 (*3*), UC/HeLa 5 (*5*), and UC/Leu 2 (*Leu 2*) was carried out in duplicate using ^{125}I-TNF and 2×10^5 cells in a total volume of 200 μl for 2 h at 4 °C in the absence (total) or presence (nonspecific) of 18 nM unlabeled TNF after 6 cycles of sorting. Cells were washed three times with cold RPMI, 10% fetal calf serum and specific binding determined as the difference between mean total and mean nonspecific radioactivity associated with the cells. Results confirm observations made with FITC-TNF that TNF-R was amplified in UC/HeLa 2-5, UC/HeLa 4, and UC/NG108 transfectants. Compared with controls UC/uns, no significant change had occurred in UC/HeLa 3, UC/HeLa 5, and UC/Leu 2 transfectants.

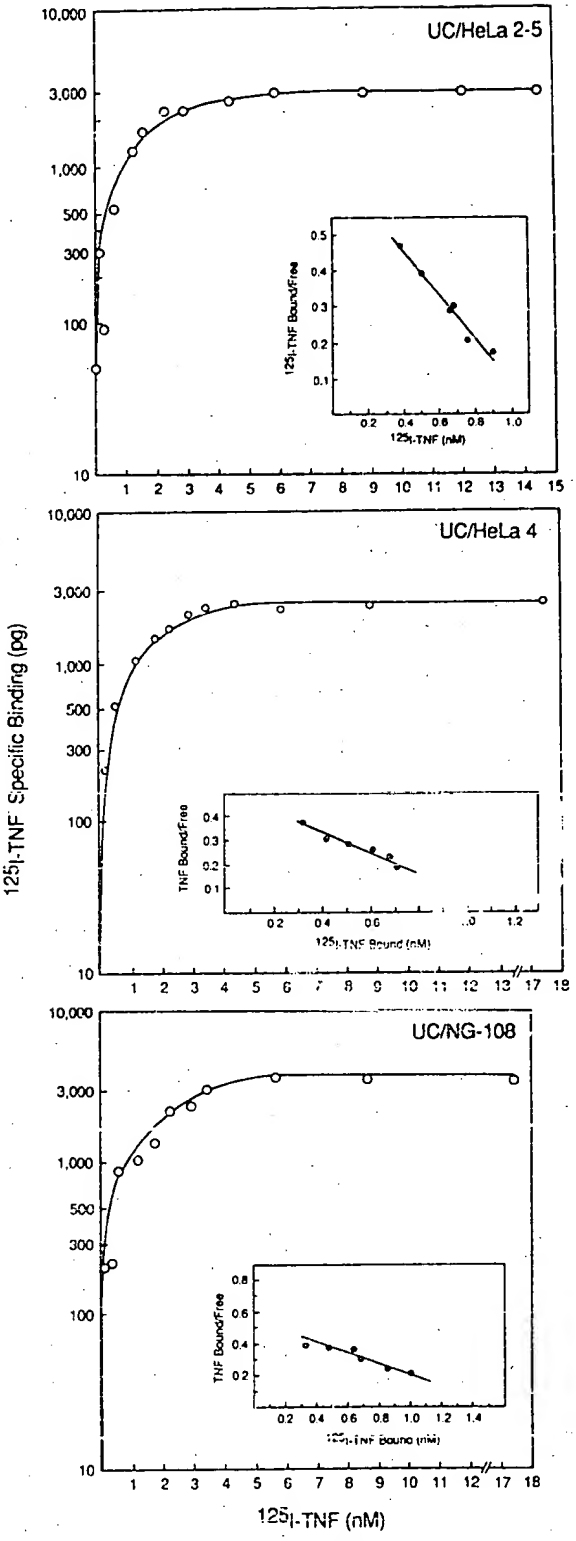

FIG. 6. **Specific binding of ^{125}I-TNF to UC/HeLa 2-5, UC/HeLa 4, and UC/NG108 transfectants.** The number of receptors per cell on the TNF-R amplified cells plus the affinity constants for receptor-ligand interaction were calculated from Scatchard analyses of the binding data. Equilibrium saturation binding to cells was carried out as described in Fig. 2. The values calculated for UC/HeLa 2-5 were 155,000 receptors/cell and K_d of 0.26×10^{-9} M; UC/HeLa 4, 144,000 receptors/cell and a K_d of 0.22×10^{-9} M; UC/NG108, 187,000 receptors/cell and a K_d of 0.28×10^{-9} M.

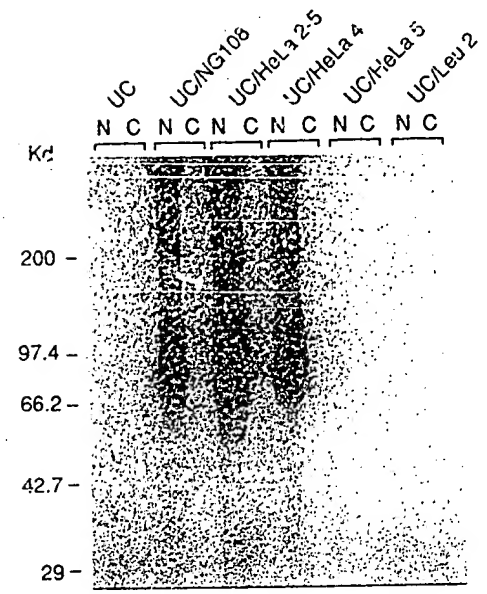

FIG. 7. **Analysis of receptor-ligand complex after cross-linking with bis-sulfosuccinimidyl suberate.** Two × 10^6 cells were incubated with 22 nM ^{125}I-TNF in the absence (*N*) or presence (*C*) of 100-fold excess unlabeled TNF and processed as described in the receptor-binding assay in the legend to Fig. 1. Cells with bound ^{125}I-TNF were treated at 25 °C with the cross-linking reagent at a 1 mM final concentration for 25 min and cells were washed with PBS three times. The cell pellet was dissolved in 0.1% Nonidet P-40 containing 1 mM PMSF. Solubilized proteins were precipitated with 10% trichloroacetic acid. The protein pellet was washed twice with 70% ethanol, dissolved in Laemmli SDS-PAGE sample buffer, and resolved in 10% polyacrylamide gel under reducing conditions. The cross-linked receptor-ligand complex was observed by autoradiography. The complex from amplified cells shown was visualized after an overnight exposure of the x-ray film. The estimated sizes of the two bands are approximately 87,000 and 104,000 daltons.

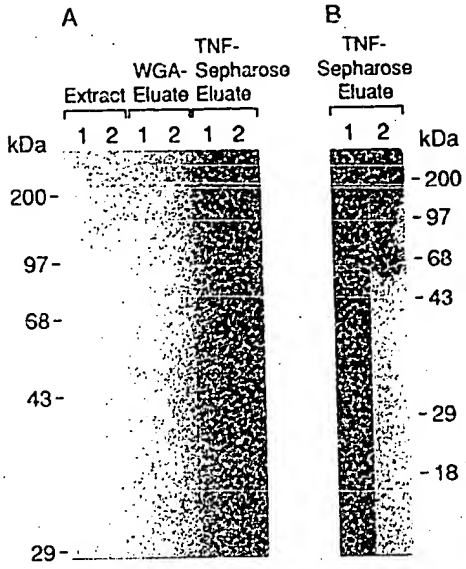

FIG. 8. **Apparent molecular weight of the TNF receptor determined by ligand-blot analysis.** *A*, detergent cell extracts and eluates from WGA-Sepharose 4B and TNF-Sepharose 4B affinity columns subjected to SDS-PAGE in 10% acrylamide gel under non-reducing conditions. Samples (25 μg of protein) were not treated with β-mercaptoethanol nor heated prior to electrophoresis. The proteins were electrotransferred to nitrocellulose paper and incubated with 8 M urea, blocked with 5% non-fat dry milk in PBS, and incubated with ^{125}I-TNF as described under "Experimental Procedures." The nitrocellulose filter washed with PBS was dried and autoradiographed. Extracts in *lane 1* were made from UC cells and *lane 2* from UC/HeLa 2-5 receptor amplified cells. The blot was autoradiographed for 20 min. A 68-kDa band with high ^{125}I-TNF binding activity is seen in eluates from TNF-Sepharose column. *B*, comparison of the sizes of the TNF-receptor from detergent extracts of UC cells (*lane 1*) and UC/HeLa 2-5 cells (*lane 2*) after WGA- and TNF-Sepharose 4B chromatography using the nonreducing SDS-PAGE and ligand blotting conditions described above, receptor from UC cells (*lane 1*) was visible as a faint 68-kDa band after 4 days of autoradiography and is compared here with UC/HeLa 2-5 receptor band (*lane 2*) as seen in a 24-h exposure.

sibly a reflection of the binding of an additional molecule of TNF to the receptor (see "Discussion"). Two faint bands of approximately the same size were also observed from extracts of UC cells but only after 3 weeks of autoradiography (data not shown).

Identification and Size Determination of TNF Receptor by Ligand-blot Analysis—Detergent extracts of UC cells and UC/HeLa 2-5 receptor amplified cells were prepared and enriched for the receptor by sequential binding to lectin and TNF affinity columns. Fractions were tested for the presence of the soluble receptor using a filter binding assay (described under "Experimental Procedures"), and aliquots were simultaneously analyzed by nonreducing SDS-PAGE and ligand blotting to determine the size of the protein associated with receptor activity. An approximately 68-kDa band with a high level of ^{125}I-TNF binding activity was recognized readily in pH 2.5 eluates from the TNF affinity matrix when using UC/HeLa 2-5 cell extracts (Fig. 8*A*). Parallel samples obtained from unamplified UC cells processed through the lectin and TNF affinity steps did not bind an observable amount of ^{125}I-TNF. However, a 4-day exposure of a ligand blot to x-ray film did produce a faint band with TNF-binding activity corresponding in position and size to the 68-kDa protein seen in amplified cells (Fig. 8*B*). This binding of ^{125}I-TNF was specific since it was eliminated in the presence of excess unlabeled TNF.

Identification of a 68-kDa TNF Receptor Protein Band—Parallel preparations from UC cells and UC/HeLa 2-5 cells were processed through the same affinity chromatography procedures, and the TNF affinity eluates were subjected to reducing and nonreducing SDS-PAGE; proteins were detected by silver staining (Fig. 9, *A* and *B*) and also analyzed by ligand blotting (Fig. 9*C*). A 68-kDa protein band was recognized only in receptor amplified cells and was not detectable under these conditions in UC cells. Furthermore, this band corresponded in size and mobility with the 68-kDa protein exhibiting high TNF-binding activity as assessed by ligand blotting. Based on this evidence we infer that the 68-kDa protein is the TNF receptor.

EBO-pcD Plasmids in Receptor Amplified Cells and Their Rescue—Low molecular weight DNA was isolated by the method of Hirt (33) or its modification described previously (29) from cells enriched in TNF-receptor after four, five, or six serial flow selection cycles. No plasmids were recovered in *E. coli* from any of these cell lines after many repeated attempts. A conclusion to be drawn from these experiments was that the introduced plasmids no longer existed as recoverable episomes. The plasmids may have integrated or rearranged such that they could not be propagated in *E. coli*. Analyses of total genomic DNA from positive and negative cell lines were made after digestion with *Eco*RI (two sites within the vector) or *Bam*HI (to release insert cDNA). Southern blots analyzed with radiolabeled probes containing EBO-pcD sequences of the EBO-pcD Leu 2 plasmid (Fig. 10) or pcD containing plasmids such as pcD-DHFR (dihydrofolate

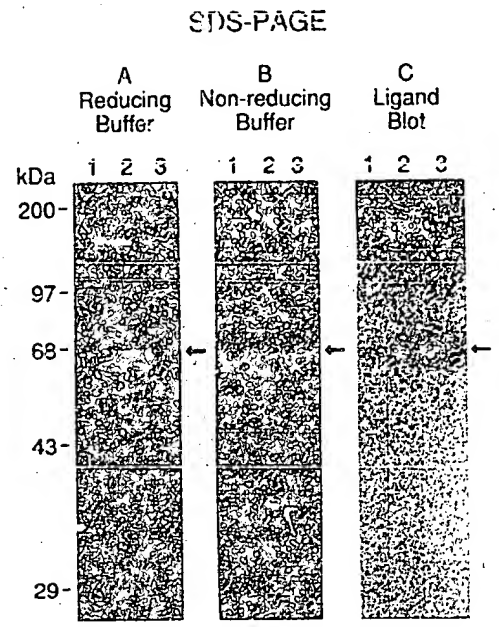

FIG. 9. **Identification of a 68-kDa protein band by SDS-PAGE and silver stain with TNF-binding activity.** Detergent extracts of UC/HeLa 2-5 cells were subjected to SDS-PAGE following chromatography on WGA-Sepharose (*lanes 1*), WGA-Sepharose followed by TNF-Sepharose (*lanes 2*), and WGA-Sepharose followed by two cycles of TNF-Sepharose (*lanes 3*). Prior to electrophoresis the molecular weight standards and the receptor samples in *A* were heated for 5 min at 90 °C in the presence of 3% (v/v) 2-mercaptoethanol and in *B* and *C* applied without heat or 2-mercaptoethanol treatment. After electrophoresis acrylamide gels *A* and *B* were stained with silver nitrate and *C* was ligand blotted with ^{125}I-TNF as described under "Experimental Procedures."

reductase cDNA inserted into the cloning site of the pcD vector (30)), or only SV40 promoter sequences (data not presented) identified plasmid sequences. Different restriction patterns were obtained from the various transfectants indicating that the integration had occurred at different sites in the genome or that rearrangements and concatameric forms were generated. The number of copies of integrated vector sequences in UC/HeLa 2-5 was estimated (by comparison of autoradiographic band intensity with known amounts of DNA) to be approximately 30 copies per cell.

One consideration for the absence of recoverable episomal DNA in transfected cells may be the duration of time between transfection and the selection of receptor enriched cells, normally a period of 6–8 weeks, during which time the plasmid could rearrange or integrate into genomic DNA. One way to reduce the probability of integration or recombination would be to decrease the time taken for selection. One such experiment is shown in Fig. 11. Here, 100 μg of EBO-pcD NG108 DNA were transfected into 1×10^8 UC cells; the cells were immediately transferred to hygromycin-containing medium and analyzed in the FACS 4 days later to measure expression from the introduced plasmids. This 4-day period was selected arbitrarily to allow cells to recover from the shock of electroporation and is comparable with a transient expression assay.

Contrary to our experience with stably transformed pools of UC cells, a distinct population of cells with high TNF receptors was observed (Fig. 11*B*, *arrow*). These cells were recovered and are referred to as sort 1B (S1B). They were transferred to growth medium and expanded into a larger population. From these, 2×10^7 cells were removed for the preparation of episomal DNA while 1×10^6 cells were cycled through repeated flow selection of the brightest 1–2% of cells. These cells remained amplified through subsequent sorts (Fig. 11, *S3B* and *S5B*). S1B cells increased in receptors 30-fold over UC/uns cells and S3B and S5B another 3-fold amounting to a total 90-fold amplification. From 2×10^7 cells of S1B low molecular weight DNA was recovered and used for bacterial transformation to yield about 1×10^5 bacterial colonies. These plasmids were expected to represent a population enriched in genes conferring TNF receptor abundance. Plasmid DNA was prepared from a pool of these transformants, and 100 μg of DNA was transfected into a fresh population of 1×10^7 UC cells which were flow cytometrically analyzed 4 days later. No increase in TNF receptor was seen in these cells after 4 days of recovery or in cells selected for high FITC-TNF binding even after seven cycles of sorting and expansion. Low molecular weight DNA was prepared from the selected cell populations and, except for S1C (see Fig. 9*E*) which yielded 20 transformants, no plasmids were recovered from DNA of any other sort upon repeated attempts. Restriction endonuclease digests of plasmid DNA from S1B and S1C with *Bam*HI of *Xho*I to release insert cDNA showed that the insert size was either very small (less than 500 base pairs) or absent. Transient expression assays have also been performed with other cells such as COS-7 and 293S as recipients for HeLa and NG108 cDNA libraries. In these experiments no receptor enriched cells were obtained upon initial transfection nor repeated flow selection of the brightest 2% of cells. Plasmid recovery was low and repeated transfections with rescued plasmid DNA failed to generate positive cells. Plasmids can be recovered from recipient cells but their abundance declines rapidly within 2–3 weeks after transfection, and, when plasmids are recovered, they have either very small cDNA inserts or have no detectable inserts (data not shown).

DISCUSSION

The diversity of biological effects induced by TNF is initiated by its interaction with cell surface receptors. The nature of the signals transduced by this interaction is not yet known, but, as a step toward understanding the signal transduction pathways, a knowledge of the receptor is required. Receptors for TNF are found on most cell types but their number is usually in the range of a few thousand per cell (7, 9–12). Because of its low abundance, purification of the receptor is a difficult task. To overcome this limitation we have combined the expression of transfected cDNAs with a powerful selection using the FACS to identify rare cells expressing high levels of receptor. Using this approach we have succeeded in isolating UC cell lines from both HeLa cDNA and NG108 cDNA transfected cells that express TNF receptor at levels 150-fold higher than untransfected UC cells. The results of the HeLa cDNA transfections were particularly interesting since only one size fraction of the cDNA library, that with 1.6–2.9-kb inserts, conferred increased receptor density in two separate sets of independent transfections. These observations suggest the presence of a specific cDNA sequence in the HeLa cell library responsible for the increase in receptors. However, the mechanism by which receptor numbers are increased is not clear. The selection pressures that were applied to select and maintain stable transformants were maintenance on 200 μg/ml hygromycin and flow cytometric sorting for high TNF receptor levels. The cDNA responsible for conferring this phenotype could possibly be a full length sequence encoding the receptor, integration and/or amplification of which causes receptor numbers to increase. It could also be due to a partial cDNA sequence whose insertion into the resident TNF receptor gene followed by amplification could lead to higher levels

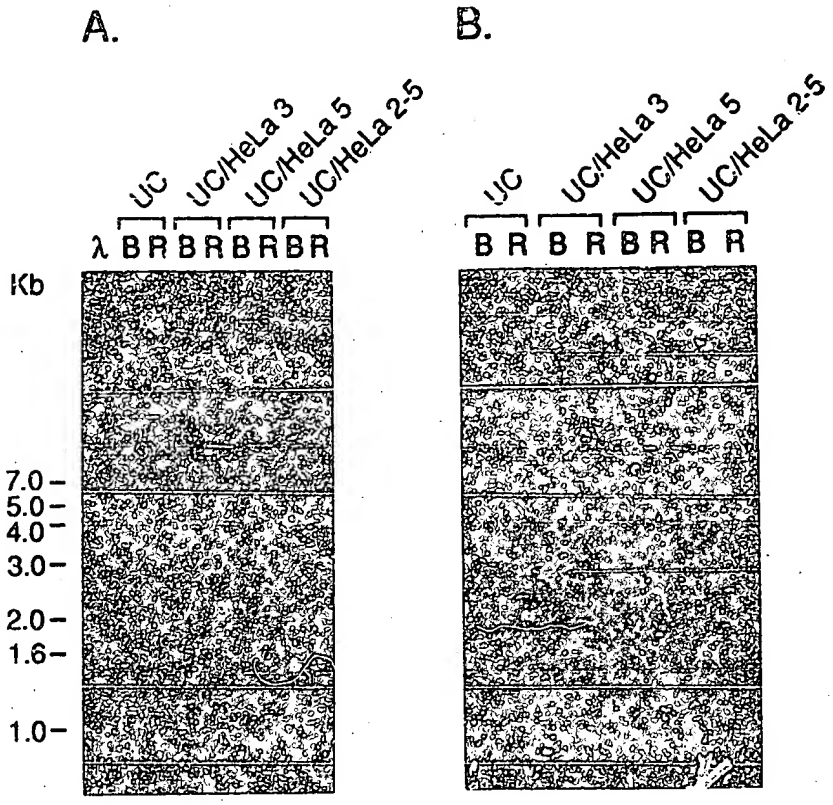

FIG. 10. **Analysis of vector DNA in cell transfectants.** Genomic DNA was prepared from different cell lines and digested with either *Bam*HI (*B*) or *Eco*RI (*R*) endonucleases and separated by electrophoresis in 0.8% agarose gels, stained with ethidium bromide to visualize DNA, and then transferred to nitrocellulose and hybridized to radiolabeled probe. *A*, ethidium bromide-stained gel showing restriction endonuclease-digested DNA and in the left margin the positions of the size markers. *B*, corresponding Southern blot hybridized with EBO-pcD, specific DNA probe containing pBR, SV40, hygromycin, *ori*-P and EBNA-1 sequences minus a cDNA insert to locate sequences homologous to the Epstein-Barr virus or pcD-derived vector DNA. UC cells are EBV-transformed and contain unintegrated EBV-sequences in the genome.

of expression. Alternatively, we may not be selecting a receptor encoding gene but one whose protein product could upregulate receptor numbers. Such a cellular product, *e.g.* interferon-γ, has been reported, although the magnitude of increase by it is a modest 2–3-fold (15, 17, 22).

Scatchard analysis of steady-state receptor binding with ^{125}I-TNF shows that indeed the dissociation constant of the over-expressed receptor is characteristic of high affinity receptors and similar to that of HeLa and UC cells with a K_d in the range of 0.9–2 × 10^{-9} M. Cross-linking studies were performed with the receptor-enriched transfectants using ^{125}I-TNF and the cross-linking reagent bis-sulfosuccinimidyl suberate. Cross-linked complexes were observed when ^{125}I-TNF was incubated with the cells in the absence of cold TNF and followed by treatment with the cross-linking reagent. Two protein bands were observed in gels at positions corresponding in size to 87 and 104 kDa consistent with previously observed bands (11, 13, 16, 23). Neither band was seen if cross-linking was performed in the presence of cold TNF. It appears that the molecular mass of the receptor observed in our studies is approximately 68–70 kDa if these complexes represent the binding of TNF monomer and dimer, respectively. However, if the complexes represent binding of a dimer and timer of TNF (27, 28) then the receptor itself may be 50–55 kDa.

Given the high concentration of the receptor in amplified cells we have undertaken to characterize its size and ligand binding properties in order to eventually purify the protein to homogeneity. An assay developed to measure ^{125}I-TNF binding to soluble receptor was useful in pursuing the enrichment of cell extracts for receptor protein. The applications of immunoaffinity chromatography including WGA-Sepharose and TNF-Sepharose (details to be published elsewhere) provided a rapid and convenient method of increasing receptor content that has allowed us to identify by ligand blotting techniques a band of approximately 68 kDa with high level ^{125}I-TNF binding activity. Nonreducing and reducing SDS-polyacrylamide gels (stained for protein and silver) showed a 68-kDa band in enriched cell extracts of amplified cells. This band was difficult to find in comparable preparations from UC cells. Therefore, our identification of the 68-kDa protein as the receptor is based on the use of the ligand binding functional assay and the presence of a protein band coincident with the activity. The usefulness of the immunoaffinity procedures employed here brings into question an earlier published report (13) describing the failure of TNF receptor to bind to TNF immobilized to agarose and its poor recovery and lack of enrichment from lectin-affinity resins. Possibly, the rich source of receptor in the amplified cells plus the development of a solubilized receptor assay to trace the receptor has helped us to optimize the use of these affinity chromatography procedures.

The sizes of the TNF-receptor complexes are approximately 87 and 105 kDa as determined by cross-linking ^{125}I-TNF to the receptor on intact cells. The estimated size of the TNF receptor deduced from the ligand blot is 68 kDa. The subtracted difference approximates the sizes of the TNF monomer (17 kDa) and dimer (34 kDa). Although TNF reportedly self-associates into a trimer (27, 28), there has been considerable discussion as to the preferred size of the TNF molecule that associates with the receptor. Since the intensity of the 87-kDa receptor-ligand complex is greater than the 105-kDa band (Fig. 7), our data reveal for the first time that TNF binds to its receptor predominantly as a monomer and to a lesser extent as a dimer.

An important aspect of the approach we employed was the use of the EBO-pcD vector for the construction of the libraries. These libraries were initially prepared in the pcD vector containing the SV40 early region promoter and the late splic-

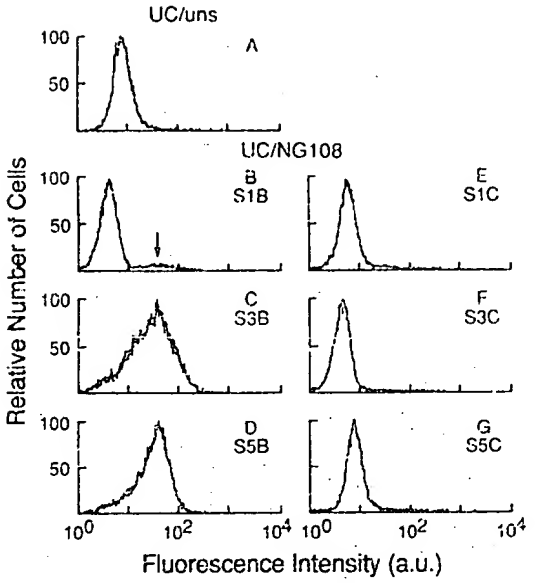

FIG. 11. **Flow cytofluorometric analysis of UC cell transfectants obtained with DNA from NG108 cDNA library or plasmids rescued from TNF-R amplified cells.** *Panel A*, control UC/uns cells stained with FITC-TNF prior to transfection to profile level of receptor expression. *Panel B*, sort 1B from 2×10^6 UC cell transfectants generated by electroporation of 100 μg of DNA from NG108 cDNA library into 1×10^8 cells after 4 days of growth in hygromycin-containing medium to reveal a distinct population of TNF-R amplified cells (*arrow*). Five $\times 10^4$ cells from this population were grown for isolation of episomal DNA and for subsequent rounds of sorting. *Panels C* and *D*, sorts 3B and 5B are from two subsequent sorts to show retention of receptor amplified phenotype. *Panel E*, sort 1C are UC cells electroporated with 100 μg of plasmid DNA obtained from 1000 amp^r bacterial colonies transformed with episomal DNA of S1B cells marked by the arrow in *panel B*. *Panels F* and *G*, sorts 3C and 5C, two subsequent sorts of the top 5% of cells from sort 1C to show lack of receptor enriched cells. *a.u.* denotes arbitrary units.

ing function (30). The novelty of the EBO-pcD plasmid lies in a sequence of DNA encoding the Epstein-Barr virus origin of replication plus the EBNA-1 gene conferring replication function to *ori*-P and a selectable marker conferring resistance to the drug hygromycin B. These genes were introduced into pcD-HeLa and pC-NG108 libraries as a cassette to transform a pcD-cDNA plasmid into an EBO-pcD plasmid, thereby conferring replication and maintenance functions on plasmids introduced into the EBV-transformed UC-lymphoblastoid cells (38). Libraries made in this vector yielded hygromycin-resistant transfectants within 2 weeks upon introduction into UC cells. When these transfectants were used to isolate autonomously replicating plasmids from cells maintained on drug selection the recovery of plasmids dropped dramatically over time. Initially, up to 2 weeks after transfection around 1000 plasmids could be recovered from 2×10^7 cells. By 3 weeks post-transfection this recovery had dropped to less than 10 plasmids and at 4 to 6 weeks post-transfection plasmids were recovered rarely. These results suggest that the plasmids were not being maintained autonomously. Restriction enzyme analyses with *Eco*RI or *Xho*I of recovered plasmid DNA showed that the cDNA insert was either missing or present as a fragment smaller than 500 base pairs (data not shown). Since the starting libraries had a wide range of insert cDNA, particularly the size selected HeLa library, this loss of cDNA indicates an inability of the plasmid to maintain structural stability or a preferential recovery of clones with short inserts. Analysis of high molecular weight DNA for vector sequences identified what appear to be integrated molecules in all transfectants analyzed. Therefore, while the EBO-pcD plasmids can be maintained as autonomously replicating units in cells for varying periods of time (29), in our experience using routine procedures they are not easily recoverable as plasmids. Instead these DNAs appear to be preferentially maintained as integrated sequences or perhaps very high molecular weight catenates that behave like chromosomal DNA. The expression in COS-7 cells of cDNA libraries constructed with the CDM8 vector (39) and affinity selection techniques using either antibody or ligand binding have worked successfully for the cloning of some cell surface and growth factor receptors (39–42). This vector or the recently described SRα vector (43, 44) offers alternative systems for cloning cDNA sequences expressing cell-surface receptors.

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Addendum—After this manuscript was submitted for publication, Smith and Baglioni (45) using ligand blotting reported 60- and 70-kDa receptors on HeLa cells. These results are partially consistent with our estimates of a 68-kDa receptor.

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